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ORIGINAL ARTICLES

EARLY SYMPTOMS AND DIAGNOSIS OF TUBERCULAR JOINT DISEASE

Read in the Section on Surgery and Anatomy at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco June 5-8 1894

BY EMMET RIXFORD, M D

SAN FRANCISCO, CAL.

In view of the fact that there are to read two papers, besides the present, on the symptoms and diagnosis of tubercular joint disease, it has seemed best to me to confine my remarks to a limited portion of the subject rather than to attempt to cover a larger field I have chosen as a fitting introduction to the study of tubercular joint disease to say a few words on the value of early diagnosis in these conditions, and the relations between diagnosis and the therapeutic measures at our command

In most medical matters it is best on general principles to make the diagnosis when the case is first seen, but this means that it is best largely as a matter of convenience and to enable the attendant through early prognosis the better to control his patient, for a little delay in instituting treatment would make almost no difference with the patient's welfare The cases are few where therapeutics are so perfected as to permit of the disease being aborted, or much modified by treatment, even if seen in the very beginning

In tubercular joint diseases, however, it is quite otherwise Here we are dealing with a disease which begins insidiously and progresses so slowly as to require observation for a considerable period of time to make that progress evident Changes in symptoms are to be noted not day by day, but week by week, or perhaps only month by month The time element here holds a prominent place

When we consider that the chief factor in the extension of the disease is traumatism more or less frequently repeated, we at once see the value of an early diagnosis, for orthopedic surgeons taking the hint have worked out the mechanical problem of how to avoid such traumatism By this, the so-called conservative method of treatment, the real cure is left to the innate resistant and reparative forces of the organism, stimulated by intelligent support of the general system If such treatment can be instituted at a time when the disease is limited to a small collection of granulation tissue, say in the articular extremity of a bone, the joint can be put in the best possible condition as regards probable cure

Experience has abundantly shown that this method of treatment when begun early gives excellent results, but it requires the constant attention of the surgeon for from two to six years, a hardship on the medical attendant, and on the parents, and most of all on the child To initiate such a course of treatment in a delicate sensitive child is like passing sentence of imprisonment for a like period of time—to be justified only by the extreme conditions of the case It were almost criminal to pass such sentence on circumstantial evidence or in fact on anything short of almost absolute certainty in diagnosis

Then again these children are mostly offspring of the poor who can not have the means to procure properly healthful surroundings and who all too often, their patience exhausted in the long siege, willfully or ignorantly neglect those measures within their reach

Were some more direct method of treatment shown to give equally good results in regard to function it would be more than welcome Of such methods there are a number which, though still in the experimental stage, bid fair to be of value

Early excision of tuberculous foci at a time when the disease is confined to small areas outside the joint proper has been long practiced and is theoretically, where it can safely and thoroughly be carried out, an ideal treatment To drill out the neck of the femur through the trochanter in coxitis, enucleating the tubercular foci in the head of the bone may be a rational procedure under certain conditions and may be carried out by those who have the requisite skill, but to be justified it must be done early and at a time when the diagnosis is most difficult Not only must the diagnosis of tubercular coxitis be certain but the lesion must be accurately located in the head of the femur The procedure is by no means an innocent one nor is it certain that in a given case there might not be left a portion of the diseased tissue Further, there may be more than one focus of disease, and of this we have no means whatever of determining the existence After all, granting that the technique of the operation can be perfected the whole matter hinges on the diagnosis Senn says that with the exception of circumscribed points of tenderness outside of the joint that indicate the existence of primary osteo tuberculosis during its early stages, we have no symptoms which enable us to make a positive differential diagnosis between a primary osseous and a primary synovial tuberculosis of a joint In the superficial joints such points of tenderness can be located, but that is quite out of the question when the focus of disease is in the hip or in the deeper parts of the other large joints However, those cases in which the muscular atrophy is rapidly progressive are quite certainly of osseous origin, or at least involve the bone

Another method of treatment and one which bids fair to be more generally applicable than early excision is puncture and injection of iodoform directly into the foci of disease This requires, with the latter, the most perfect aseptic technique, fairly accurate localization, though there is little or no harm done by injecting sterile iodoform emulsion into the tissues about a joint and if the emulsion is not known to be sterile it should not be used at all

A number of other methods have been suggested, some of more or less value, the latest of which is that advocated by Bier in the Twenty-first German Surgical Congress In a second paper in 1893 he reported forty-three cases of surgical tuberculosis treated by this method, which consists in causing a continuous hyperemia of the extremity for a number of weeks Many of the cases were cured within three months The method has been taken up by Mikulicz who has seen from it sufficiently good results to consider it of considerable value If this simple procedure proves to justify the hopes of its originator it can be tried as soon as the diagnosis is made and being thoroughly innocent the diagnosis need not be absolute

The study of diagnosis and treatment should go hand in hand Important as it is to make the diagnosis of tubercular joint disease at the earliest possible time, the diagnosis should be made certain before such severe measures as long continued immobilization or operative procedures are instituted In many cases, if immobilizing apparatus be applied early, the symptoms of muscular spasm, pain and tenderness will soon subside and unless the diagnosis was certain in the beginning, there will be a lingering suspicion that an error has been committed and the child is being badly punished In other branches of medicine in case of doubt it is generally advisable to settle upon the more severe of the diagnostic possibilities, but here a little delay sufficient to clear up the doubt is the lesser of the two evils so long as there remain to be devised methods of treatment entailing less hardship on the patient Unfortunately, too, there is no symptom or group of symptoms which determines with certainty when a tubercular joint is permanently cured The case is not unlike that of syphilis In many instances it is impossible to make the diagnosis, no matter what the probabilities, before the appearance of secondary symptoms and it is a very severe thing to initiate a long course of mercury and iodid of potash in a case of doubt So in tuberculosis of the joints, the diagnosis is not to be made in the very beginning of the disease, we must wait for certain symptoms and sometimes, be it said, characteristic symptoms are late in making their appearance

I do not wish to be understood as really advocating delay, but rather as emphasizing the necessity of making the diagnosis certain beyond reasonable doubt and then the earlier the better

We have still too many cases of rheumatism developing into hip joint disease and too many cases of hip joint disease with cautery marks and blister stains about the knee joint

In view of the previous considerations let us consider at what stage of the disease the diagnosis is to be made with certainty Taking tubercular coxitis as the type, in general the first symptom to be noted is almost invariably the limp, an evidence of more or less conscious pain There is nothing characteristic about this limp—it may be simulated by a number of other conditions Its real value is to direct the attention of the physician to the child's hip, that he may the more carefully observe the case and watch for more definite symptoms Actual pain about the hip, or pain reflected to the knee, makes the diagnosis of coxitis probable, but in many cases pain severe enough to attract attention is entirely wanting It seems to bear no definite relation to the extent or character of the lesion Of most value in making the diagnosis are symptoms ascribable to inflammatory irritation, chief of which is spasm of the muscles controlling motion of the joint No single symptom is characteristic of tubercular joint disease but the nearest approach to it is this muscular spasm, especially if it varies in intensity and intermits A case in point deserves mention

A boy of 5 years in whose family there was a case of pulmonary tuberculosis was noticed to limp for several days The right leg was held somewhat everted, slightly flexed and abducted, though the boy would stand perfectly straight when his attention was directed to his position Considerable joint pressure made in various directions failed to elicit pain, movements of the leg were normal in extent and painless, save adduction which was slightly limited, as was also rotation of the thigh in the flexed position The child was kept under observation without treatment and the limp entirely subsided in a week or two He was seen from time to time for several months but no further evidence of hip joint disease appeared

Another case in a boy of 14 whose brother came to me nearly a year ago in the so-called third stage of hip joint disease with a large abscess, has had a hip-joint limp for many months It varies in degree, subsiding almost entirely at times He has occasionally had a little pain in the region of the joint The leg is habitually held in slight flexion but abduction is more marked Adduction is somewhat limited There is no shortening There is a slight fullness to be made out about the hip Vigorous movement up to the limits of range failed to bring out expression of pain as did also severe joint pressure in various directions It was thought to be a low grade of inflammation in a bursa in the region of the lesser trochanter The boy is kept under observation and without treatment the condition has materially improved though there is still a considerable limp at times His general health is excellent and he is a lively growing boy

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EARLY SYMPTOMS AND DIAGNOSIS OF TUBERCULAR JOINT DISEASES

Read in the Section on Surgery and Anatomy, at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco, June 5-8, 1894

BY A B JUDSON, M D
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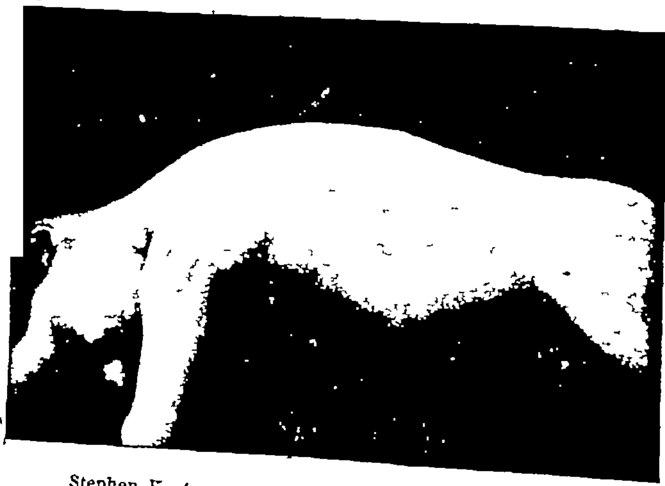
In writing on the subject proposed by our honored chairman I have limited my paper to the consideration of Pott's disease of the spine and hip disease These affections present many difficulties, especially in the early diagnosis In diseases of the other joints the same principles and methods of examination hold good and diagnosis is easier because the bones lie nearer the surface

POTT'S DISEASE OF THE SPINE

The most important symptom of Pott's disease of the spine is pain in the stomach Two lines which should find a place in the *vade mecum* of every physician are "The pain of spine disease is in the stomach," and "the pain of hip disease is in the knee" No prescription for recurring colic should be written, unless it is preceded or presently followed by a careful questioning of the health of the spinal column The inspection thus prompted may reveal a projection in the median line which should be located by counting the spinous processes downward from the vertebra prominens (seventh cervical), or upward from the fifth lumbar, which is in the line connecting the posterior superior spinous processes, or the result of one method may be verified by the other The rounded back of rickets or spastic con traction should not be mistaken for the deformity caused by vertebral caries In the lower dorsal region, from the sixth to the ninth inclusive, it is also well to avoid a peculiar source of error The spinous processes of these vertebræ have a great inclination downward, overlapping like the shingles on a roof, and when a thin patient stoops they approach the horizontal, and pushing against the skin make a projection which has often led to error or unnecessary apprehension This mistake may be avoided by noticing whether the projection is angular or not

The expression, *angular curvature*, has been criticised because an angle and a curve are essentially different But in practice the term is admissible and convenient, because the long natural curve of the spine is broken in Pott's disease into two short curves which are united and make an angle at the point of union, as shown in the figure This point may not make a marked projection, but if it marks the union of two curves even in the slightest degree, as shown in the figure, it means that caries is present and has proceeded to very serious destruction of the bone A moderate lateral curvature is also sometimes present An angular curvature then is usually an absolute demonstration of the presence of Pott's disease



Stephen K., 4 years old Pott's disease Duration of symptoms four months Normal curve broken into two curves united at an angle

But it is sometimes desirable to make or approximate a positive diagnosis before angular curvature occurs, and to do this several things are to be borne in mind The first and most important has already been mentioned, the pain in the stomach, or gastralgia Next in importance is the peculiar gait The child in walking avoids stamping with his heels and puts more of his weight on the toes than is customary, or he walks as if he were stepping on a surface which he fears will break if he is not careful, or the line made by the top of his head as he moves across the room is noticed to be rather a straight line than the undulating line made by the rising and falling of the head in the buoyant gait of a well child And then the deportment of the patient is to be considered He will play quietly by himself perhaps, or, easily tired he will frequently lean across the mother's lap, or if the caries is at a high level he will often support his head with his hand the elbow resting on a chair or table He is disturbed by the jolting of a carriage or street-car He will sometimes be stopped in the course of a rough game by a seizure of pain in the stomach, the laughter ending in crying A common sign is a frequent or habitual grunt accompanying each expiration By following these lines of observation a conjectural or rational, and sometimes a positive diagnosis may be made before angular curvature appears

In making a diagnosis but little attention may be paid to the general condition Many cases are seen in which the health remains good in the early stage, as is shown by the normal appetite and digestion, abundant fat, and good facial color and expression While these signs of health are present and persistent the disease in question may be in quiet pursuit, and this insidious quality should not be forgotten

In two points, thus far, we have seen that the unexpected has claimed our attention The pain is in the stomach and not in the back, and the general health shows, as a rule, no reaction But there is another surprising thing to be noticed in the fact that, although the patient's back is virtually broken, local disability, which is usually found in parts which have lost their bony integrity, is almost entirely absent So true is this that when we hear of spinal pain and disability we at once think that there is no Pott's disease If these alarming symptoms are present, together with a comparatively frank onset, it is necessary to think of cancerous disease of the vertebræ, or paraplegia dolorosa I have seen two or three verified cases of this disease which were believed at first to be Pott's disease and other cases are in my memory which, with a fatal termination unexplained in the absence of autopsies, may have been instances of the rarer and more intractable affection

HIP DISEASE

Two precepts which should be deeply inscribed in the memory are "The pain of hip disease is in the knee," and "the pain of spine disease is in the stomach" Recurring pain in the knee, in the absence of physical evidence of disease in this joint, should call early attention to the condition of the hip But the pain of hip disease, except as an alarm, is not often an important element in diagnosis It belongs to the group of subjective symptoms and may be almost disregarded in favor of objective signs in an affection which displays so many physical evidences of its presence Among the first signs is lameness which, in the early stage, may disappear entirely to return after an interval of days or weeks, it is present sometimes in the morning when the patient leaves his bed, and "wears off" after a brief period of activity, it breaks up the natural rhythm of walking, in which equal time is given to the two feet, leaving the well foot on the ground longer than the affected foot and leading the former to give a more accented stroke as it hastens to relieve the latter from the weight of the body Akin to lameness is the attitude "at rest" in which the patient stands favoring the affected limb, which is abducted and advanced while the weight of the body is principally thrown on the well limb

Next to lameness in the order of obviousness is the muscular atrophy, seen in the flattening of the nates, as the patient stands with his clothes removed, and in the characteristics of the gluteal fold, which is shorter and more shallow than that of the unaffected side, and recognized by the tape measure which shows that the thigh and leg are less in circumference than those of the well side

Then comes the most valuable, and yet most frequently neglected, sign of the early stage, the checking of passive motion by reflex muscular action This is found earliest perhaps in rotation Let the patient sit on a table with the legs hanging over the edge and then impart a lateral pendulum like motion to the foot and leg and note whether the arc of motion is less on the suspected side Or let the patient lie supine on a hard bed or table and grasp the limbs, giving them a rolling motion in inward and outward rotation On the well side the inner and outer borders of the foot will in turn strike the table, or nearly so, while on the suspected side rotation may be obviously limited Limitation of abduction, adduction and flexion may be sought in a like way by testing the passive motion of the two sides alternately The patient may be induced to [illegible] a test for limited passive flexion by [illegible] the chin and kissing the knee On the suspected side he may not be able to bring the knee to the mouth These examinations should be made [illegible] and the utmost gentleness, for our [illegible] to detect very slight differences in muscular [illegible] to recognize the reluctance of the [illegible] certain directions, although they [illegible] their spasmodic action prevent wide [illegible]

[illegible] from this reflex interference with passive [illegible] instructive to note the deportment of the [illegible] as revealed by the hand placed on [illegible] may be entirely relaxed, but when [illegible] begun they may suddenly contract [illegible] in spasm till the attempt at passive [illegible] and the limb is allowed to remain [illegible] Or the abdominal muscles, as well as the adductors, may make a single reflex spasm the moment [illegible] motion is begun

In this, the early stage, all these reflex signs should be sought for in both limbs for the sake of comparison By comparison, too the inguinal fold (as well as the gluteal) may be seen to be short and shallow, or the surface over the capsule of the joint may be slightly elevated, or the soft parts about the trochanter may, from being infiltrated, have a brawny feeling which makes it difficult to include a small portion of the skin and cellular tissue in a pinch between the thumb and finger

When a later stage is reached some of these points may be dismissed from consideration because overshadowed by three unmistakable and easily read signs, and a comparison may not be strictly necessary between the two sides, although a careful comparison is useful at any stage as throwing light on the progress or severity of the case 1, the patient's lameness is then constant, 2, the muscular disparity is marked, being due in part to over-work on one side and disuse on the other, and 3, there is no motion or almost no motion in the joint These three features combined make a picture of hip disease which is not easily mistaken for anything else In regard to the first and second, nothing need be said, but the absence of motion in the joint may readily escape detection The amount of motion, or its absence, can be recognized only by noticing the deportment of the pelvis while attempts at passive motion are made To test for lateral motion, arrange the pelvis so that the line of the two iliac spinous processes is at right angles with the edge of the table or the wall of the room, then if there is no motion in the joint the slightest attempt at passive motion in abduction or adduction, will be accompanied by a disturbance of this line and, if there is some motion, its extent may be observed by noting the point at which the line is disturbed by either abduction or adduction To test for antero-posterior motion arrange the pelvis by raising the limb till the lumbar spines rest on the table and then as flexion or extension is attempted, in passive motion, the disturbance of the pre-arranged relation between the lumbar spines and the table will indicate the absence or the extent of antero-posterior motion in the joint

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THE EARLY SYMPTOMS AND DIAGNOSIS OF TUBERCULAR JOINT DISEASE

Read in the Section on Surgery and Anatomy at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco, June 5-8, 1894

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No more important division of our subject could have been given me for consideration than this, for while the aim of our calling is to cure disease, yet preceding the therapeutics comes the definite recognition—the diagnosis And it is just here the physician most frequently errs Four-fifths of the cases of joint and spinal disease that come under my observation have previously been wrongly diagnosed by medical men It is in the matter of diagnosis that we need to more thoroughly enlighten the profession—how to early and unerringly recognize these joint troubles To those of us who have given years of study to this subject, it seems simple and trite, and so while to some I may be amenable to the charge of being commonplace, yet in accordance with the duty assigned me, I will briefly outline the symptoms of the tubercular joint affections

The earliest symptom, and one ever present, is limitation of the normal motions of the joint It may be semi-voluntary from apprehension of pain, but as a rule it is caused by the reflex contraction of the muscles controlling the joint We assume it granted that there is an inflammation in some or all of the tissues composing the joint, and as the nerves supplying these tissues also have filaments from their main root distributed to the muscles, so reflexly the latter contract when the former are irritated Within a limited radius motion is permissible—beyond that, both in flexion and extension, hindrance is met A stiffness more or less pronounced is had, observable not only in the freely movable joints, but even in the spine, as is so pronouncedly observed in Potts' disease This muscular contraction with limitation of motion continues so long as joint irritation or inflammation exists If it is a joint of the inferior extremity that is affected, limping or lameness is produced and is the symptom first observed, and which calls attention to the afflicted child

The position in which the limb finds itself as a result of the joint inflammation next deserves attention Ordinarily, yes, universally, it is flexion The position of the foot in ankle joint disease would seem to be an exception, as it is always carried downward—but what is that but palmar flexion? Is this flexion of the diseased joint due to the patient voluntarily placing it in the position of greatest ease? Is it because the flexor muscles are more powerful than the extensors, and as all are goaded to contraction they overcome their antagonists and thus flex the joint? Or is it because that portion or tissue of the joint which is diseased is supplied with the same nerve or nerves which are distributed to the flexor muscles, and thus the latter by reflex irritation contract? Or rather may it not be that the joint in its dilemma simply assumes the position which is most natural to it, that is if it is a question simply of flexion and extension? We always find the fetus with marked and the young child with decided joint flexion.

Scarcely a case will go through its course without pain, most marked when the initial lesion is in the bone, and too, when the progress of the case is acute, usually confined to the joint, occasionally, as in coxitis, remote. The night cry is characteristic of these affections, being most marked during the early hours of slumber.

Swelling of the joint is a decided symptom, more especially so as the case progresses, exceptionally so, however, in *caries sicca*. The joint enlargement is more pronounced when the synovial membrane is the seat of the inflammation than when the initial lesion is in the bone. The superficial joints evidence the swelling more promptly than the deep seated articulations, such as the hip. The thickening of the capsule and of the periarticular structures, with exudation in the latter, and also effusion within the capsule cause the swelling.

Nineteen-twentieths of the cases occur in children, the growing epiphysis being especially vulnerable to the bacillus. In the adult the synovial membrane is more prone to be the seat of the disease.

Dislocations and marked deformities occurring during the progress of the disease do not concern us here as we are speaking only of the early symptoms.

The tubercular troubles need not be confounded with other articular affections when we consider their frequency, their proneness to attack children, their insidiousness and their slight influence on the general health in the early stage. Ordinarily unarticular, *i. e.*, two or more joints, being rarely or never attacked at the same time. A tubercular history, inherited or acquired, would assist in the diagnosis. A suspicion of syphilitic joint disease could be cleared up by a course of specific treatment. Abscesses do not occur early, but a rheumatic or gonorrheal synovitis would be attended with more pain and more acute symptoms than a tubercular abscess. Doubt as to whether there was fluid in the joint and also as regards its character could be determined by the tapping needle. Rheumatism may attack children but likely as a polyarticular affection and with fever. The spine and joint affections following typhoid fever are ephemeral and quite amenable to treatment by rest, and thus the differential diagnosis of tubercular joint troubles is readily made out.

THE CONSERVATIVE TREATMENT OF TUBERCULAR JOINTS

Read in the Section on Surgery and Anatomy at the Forty-fifth Annual Meeting of the American Medical Association, held at San Francisco June 5-8, 1894.

BY REGINALD H. SAYRE, M.D.

NEW YORK.

The chairman of this Section has asked me to prepare a paper on the conservative treatment of tubercular joints and this brings us to the question of what is conservatism. According to the views held a few years ago in Europe and even now upheld by some, conservatism consisted in the immediate eradication of all foci of tuberculosis as soon as detected, on the ground that each focus was so dangerous a menace to life that it should be treated with the same kind of conservatism we would use in dealing with carcinoma. According to the observation of many men of vast experience in this country, however, this view is entirely too radical and the etiology of tubercle, both in joints and in other parts of the body, shows us that better results are to be obtained by less radical modes of procedure. For instance let us suppose we have a bone infected with tuberculosis in the neighborhood of a joint, for it has been shown that tuberculosis of joints in the great majority of cases commences in the bone although in a certain number of instances, it may primarily affect the synovial membrane; let us suppose, I say, that we have a tubercular focus, in order that this tubercular disease shall spread, it not only must have its germ, but it must have the proper soil in which the germ can grow and the proper conditions favoring its development and conservative treatment in my opinion consists in so regulating the conditions of life of the patient and the conditions of the affected joint that they are not favorable to the growth and development of disease, and if we can so regulate the conditions which surround a patient's life, that the elements necessary for the growth of tubercular bacilli are not present, the patient will recover. We see numberless instances of patients who have been practically left to nature, and who have gotten well, gotten well, however, as a usual result with deformity and disability. But if the vitality of the patient was sufficient to enable him unaided to recover with deformity, properly applied surgery should have enabled him to recover without deformity in most instances if his vitality was sufficient to enable him to recover at all.

What are the elements necessary to the preservation of a joint that has become the seat of tuberculosis?

1. The prompt recognition of the disease in its earliest stages. This is the most important consideration in securing a good result, and one which can not be under estimated.
2. To give the joint physiologic rest as soon as the slightest symptom of disease is detected.
3. The protection of the joint from traumatism.
4. The continuance of this protection for many months until all symptoms of disease shall have passed.
5. Building up the general health of the patient to the highest possible degree.

These general principles would, I presume, be accepted by most of us, although there might be some differences of opinion in the practical application of methods to secure these results.

The first thing that should be done when a patient comes under observation with a tubercular joint is to put that joint, as nearly as possible, in a condition of physiologic rest and to do this at once. A slight delay at the onset of treatment may mean months and years of subsequent suffering to the patient, which might have been avoided by the prompt application of measures cutting short the inflammation at the start. It is not sufficient, as a rule, to tell a patient with an inflamed joint to go to bed and keep quiet. If it is a child, it will not have intelligence enough to keep quiet, and even if it had

is too short, and I believe that if instead of two years the patient were told in the beginning to expect three years of treatment, it would be nearer the truth There are cases of tubercular joint disease which get well inside of a twelvemonth, but they are largely in the minority, and so exceptional that the patient should not be led to expect anything of the kind when an opinion is asked as to the length of time during which treatment will be necessary I think that the severity of pain in the beginning or the rapidity of the onset of the disease can be used as a very accurate guide in estimating the duration of the process, for those cases which seem the simplest when they first come under observation very often are the most tedious to manage

The question of effusion into joints is a very important one, and the wonderful benefit to be derived from rest and compression is something very surprising to those who witness it for the first time In cases of acute synovitis, aspiration is often indicated, but in the chronic tubercular joint, compression, with a large compress sponge wrung out in cold water, and bandaged very snugly with a cheese-cloth bandage, is to be preferred

The question of abscesses is one which comes within the scope of one of the other papers, but I would say that there is also a conservative side to the treatment of abscesses If the abscess is simply a collection of tubercular material, I believe that it is safer to let it alone and treat it by compression, unless it is in a position where the focus of inflamed bone which gave rise to the abscess can be removed with safety without sacrificing too much healthy bone in the operation If, however, the tubercular mass becomes infected with pyogenic organisms, the case is altogether different and demands prompt surgical interference

The time limit which has been set by your chairman is so short that it will be impossible for me to enter into details in regard to the practical application of the principles I have just mentioned, in their relation to the different joints of the body except in the most cursory way, but I will crave your indulgence for a few moments while I rapidly mention a few points which have seemed to me to have been overlooked quite frequently by the general practitioner

In treatment of the spine, for instance, in the case of children under 3 years of age, as a rule it is impossible to apply any apparatus which will allow the child to run about, and secure for its inflamed spine the rest which scientific treatment demands, and yet I have frequently seen children between 1 and 2 years of age to whom plaster jackets and various other kinds of apparatus had been applied while the child was allowed to run about These children should always be treated in the recumbent position, the weight of that part of the body which lies above the point of disease being in this way removed and at the same time gentle traction should be made upon the head, while counter traction is made upon the feet or pelvis, for the purpose of counteracting the reflex muscular spasm which is always present in inflammation of any joint These principles can be carried out if the child is placed upon a hard bed and securely bandaged to it But it is extremely difficult to keep the child in a proper condition of quiet on a bed of this kind and it also has the great disadvantage that the child can not be carried up and down stairs, and given the benefit of fresh air and outdoor life without being removed from its bed, which proceeding is necessarily attended by more or less traumatism, and I therefore prefer to put the child in a wire cuirass or fasten it to a board, like an Indian pappoose, if it is too poor to buy anything better In fact, you practically have a portable bed made but a little larger than the child and the child and bed can be carried downstairs, placed in a rubber-wheeled baby carriage and transported wherever its health demands In many cases of older persons this recumbent position is far better treatment for the spine than any other, but it is so difficult to carry a child about when it is 5 or 6 years of age, that it is almost always treated with some kind of an appliance which allows it to run about In the vast majority of cases I think that the best appliance for children of this kind is the plaster of-paris jacket combined with the jury-mast, but it is not the simply wrapping a plaster bandage around a child which does it good, but the correct application of the principle which I have sketched and having a child's spine at rest, whether it be by a plaster bandage or an iron splint, or any other material that the surgeon chooses to adopt which benefits the patient, and to simply envelope it in a loose plaster bandage which slides up and down and does not protect its spine, but is merely an additional weight, hanging upon its already enfeebled form (in some cases actually held up by shoulder straps instead of resting on the ilia) this is the treatment which I wish most strongly to condemn In order to support the spine a plaster jacket must have a basis of support, it can not be applied to small children with undeveloped hips, and in applying it the child must first be stretched to the point at which it feels comfortable, and plaster bandages fitted so accurately that the child is retained in that position twenty-four hours a day, the jury-mast being added if the disease is above the tenth dorsal vertebra, in order to effect this object As long as a child has a grunting respiration, a tender careful gait, and desire to support itself against any object which comes within its reach, just so long is its back imperfectly guarded by the apparatus which it carries, and those children should be taken off their feet, put to bed, and absolute rest of the inflamed bones maintained for a long period of time

In the hip joint also, I frequently see efforts at treatment of no avail, because the gentleman who has had charge of the case has thought that the application of this, that, or the other splint was all that was needful to effect a cure, whereas it is the proper application of the principle of rest of the inflamed joint which is essential, let the principle be carried out in whatever way best suits the surgeon

I have seen traction applied to inflamed hips, flexed, and strongly adducted, with the result of causing intense pain to the patient, simply because the traction was not applied in the proper direction, and because the patient's body was not held firmly fixed in bed, in order that the traction might have some point from which to pull I have seen splints put on patients' limbs in this crooked position, which were only meant to be applied to the body when the legs were straight and parallel, and which under these circumstances produced great pain and suffering

In these distorted and inflamed joints, the first thing to do is to immobilize the body and sound leg in the straight position, and allow the to assume such attitude as it will while the spine is flat, and a line joining the anterior superior spines of the ilia is at right angles to a line passing from the center of the sternum to the symphysis pubis Now, make slight traction on the diseased limb, in the direction in which it assumes while the trunk retains the position I have just mentioned, and make the traction just sufficiently strong to give ease and comfort, the leg being placed meantime on an inclined plane to give it support If you have fastened the patient securely in bed, and applied traction as I have indicated, you will make him comfortable and can gradually change the direction of this traction little by little, day by day, until both legs are flat in the bed and parallel, without tilting of the pelvis or arching of the lumbar spine If you find that traction in the line of the deformity does not give the patient absolute comfort pass a bandage around the thigh, and make a slight lateral traction outwards In some cases [illegible] to the joint which the longitudinal traction fails to give, but is not necessary in all cases When the legs are parallel apply your hip splint, and [illegible] going to again allow the patient [illegible] while the inflamed joint is [illegible], but if you find that the patient begins to [illegible] in his joint when you allow him to get [illegible] on his splint, or splint and crutches, it shows that [illegible] applied the splint in such a manner as to protect the joint from the traumatism of locomotion [illegible] if you cannot succeed in so adjusting it that the child can go in the open air without increase of [illegible] in its joint and without being disturbed at night, put it back in bed, and have it [illegible] in the air in a baby carriage until you [illegible] of so applying the splint as to protect the inflamed joint and, at the same time, leave the rest of the body free for locomotion

In passing to the knee I would draw attention to the fact that the knee is not simply a hinge joint, and that efforts to straighten a knee which has taken on the characteristic deformity of chronic tubercular inflammation of the knee-joint, by simply pulling on the leg are apt to increase the pain, because the front of the knee joint is strongly compressed by force applied in this manner There is always a sliding backward of the head of the tibia upon the condyles of the femur in cases of chronic knee-joint disease, and to counteract this posterior displacement of the tibia, pressure must be exerted on the head of the tibia from behind forward, at the same time that traction is made in the long axis of the shaft of the tibia, if we wish to relieve the spasm of the joint, and correct the deformity of the knee The tight ham-string muscles, brought into fierce spasms as they are by irritation within the joint, form a fulcrum, and when the knee is straightened as if it were a simple hinge the joint surfaces are pressed together with a lever of enormous strength acting upon these firm ham string muscles as a fulcrum, which increased with articular pressure results in pain and damage to the joint On the contrary, by applying traction for the purpose of reducing the deformity along the line of the articular surface of the condyles of the femur, the deformity is corrected gradually, almost imperceptibly, and the joint can then be put up in a splint which embodies the same principles of treatment—traction in the long axis of the tibia, and pressure from behind forward against the head of the tibia, while at the same time the patient is prevented from bearing weight upon the inflamed joint, and able to get the benefits of fresh air and outdoor life during the months or years of treatment which will be necessary before the joint is well

Your chairman has warned me that ten minutes is to be the limit of our papers, so all that I can hope to do is to draw your attention to the principles of treatment and leave the practical application of them to some future occasion

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THE CONSERVATIVE TREATMENT OF TUBERCULAR JOINT DISEASE,

OR, MORE EXPLICITLY, THE PURELY CONSERVATIVE TREATMENT OF TUBERCULOSIS OF THE JOINT STRUCTURES

Read in the Section on Surgery and Anatomy at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco, June 5-8, 1894

BY HARRY M SHERMAN, M D
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For the purpose of the present paper it does not especially concern us which or how many of the different tissues which together constitute a joint are affected, and I shall not attempt to discuss methods of treatment based on anatomic differentiation of the location of the lesion It may, however, be stated that in the great majority of cases the initial lesion, so far as the joint is concerned, is in the bone, but in the purely conservative treatment the management of these cases differs hardly any from that of those which are primarily synovial

Underlying the treatment of all forms of chronic tuberculosis, of whatever tissue or wherever situated, is the gospel of rest and food This is founded upon the fact, learned in the first place by experience, and confirmed later by the results of biologic investigations, that there is in living tissues an inherent antagonism to all forms of infection, and that this antagonism is best exerted by tissues that are not functionating and are at the same time well nourished In the infection of no tissue has this been better shown than in the infection of bone This is due, in part, to the very chronic course these cases follow, which permits careful and extended study of them, and in part to their large number—for tuberculosis of the joints or bones constitutes more than 80 per cent of all cases of tuberculosis in patients under 17 years of age, and I have calculated on reliable data that in San Francisco, for instance one child out of every 286 of the child population has tuberculosis

Work, for a bone, is resistance to pressure and strain,—pressure being produced by the weight of carried structures, and strain caused by the use of the bone as a lever As bone is a living tissue, work must physiologically be followed by a loss of energy and the need of rest Rest for a sound bone necessitates that it shall be subjected to neither pressure nor strain beyond that which properly exists between the different parts of the skeleton with the individual quiet Perfect rest for a diseased bone contemplates that it shall be subjected to absolutely no pressure or strain, whether that be developed in the doing of work, or be the natural result of its position in the skeleton Practically, it is impossible to wholly eliminate the latter factor, and the aim of treatment on conservative lines is to secure, in the first place, the complete cessation of active work and then to lessen as much as possible, the physiologic and unavoidable pressure between bones so that a rest of therapeutic value may be had During the period of this rest careful attention is given to the general health of the patient, and every effort is made to secure a gain in body weight This is a brief statement of the principles of conservative treatment

There are two major plans, in accordance with one of which these principles are applied One aims to secure rest by fixation alone of the joint, claiming that with fixation is absence of inter-osseous friction and of pressure This is considered to be true, no matter what may be the relative position of the bones, but the plan arranges first for the gradual correction of any faulty position and then fixation in the proper position So long as a pathologic process is present the fixation must be maintained, and *never* must the joint be released until the reparative process is complete In this method no account is taken of pressure developed by the muscular spasm which is the most common and prominent accompaniment of joint lesions

The other plan endeavors to secure rest by traction below and counter traction above the diseased joint, thus addressing itself primarily to the spasmodically counteracted muscles, by antagonizing their spasm, relieving the pressure on the bone and consequently the cause of the spasm itself, the rest *from* the spasm and the spasm-pressure removes the tendency to early deformity, and the immobilization is then an easy matter,—for in a diseased joint there is no tendency to ordinary motion, but rather the avoidance of it In regard to arranging for the gradual correction of any faulty position and to the continuance of treatment until the disease has ceased and repair is accomplished, this second plan does not differ from the first

Of these two distinct methods of attaining the same end, the latter apparently complies more nearly with the requirements of the proposition

Under conservative treatment, cases of joint disease follow one of three courses Either they entirely and wholly recover, with a joint so little damaged that its function is in no ways impaired, and its disability must be looked for to be found, or they recover after a certain amount of permanent damage has been inflicted, are functionally more or less incompetent, and their disability is evident, or they do not recover but go on for long periods of time, their condition constantly bad or going from bad to worse, they can perform no function, their disability is complete

In its results then, tuberculosis of the joint structures differs in no particular from tubercular or other forms of not necessarily fatal infection of any other part of the organism That is, the conservative treatment is a method of treatment but it is not a means of cure

It is important, for its proper use, to know in what cases it will succeed and in what cases fail

At the Twenty-third Congress of the German Chirurgical Society held in Berlin on April 18 of this year Bruns, of Tubingen, reported 600 cases of tuberculosis of the hip joint, of these but 200 were available for statistical use They covered a long period of years, had been treated by various methods all conservative, as by revulsives, vesications, cauterizations, immobilization, traction and iodoform On their results he declared himself, with very slight qualification in favor of conservative treatment He summed up practically, as follows

1 Of all cases, 48 per cent begin in the first ten years of life, 37 per cent in the second ten years, and but 6 per cent in the third ten years

2 In 66 per cent of all cases there is suppuration, abscess and fistula

3 Recovery is attained by conservative methods in 55 per cent of all cases, and in an average time of four years

4 Death occurs usually of a visceral tuberculosis, or amyloid degeneration, or sepsis, in 10 per cent of all cases, and in an average time of three years

5 Prognosis depends chiefly, in each particular case, on the presence or absence of suppuration—of non suppurative cases 77 per cent recover, of suppurative cases 42 per cent only

6 Prognosis in general depends on the age of the patient when attacked In the first ten years of life the recoveries are 65 per cent, in the second ten years, 56 per cent , in the second twenty years of life, 28 per cent only No case recovers which is over 40 years old when attacked Suppurative cases are especially serious in patients over 20 years

7 *Most of the* recovered cases die later of tuberculosis of other organs Of those who recover in the first ten years of life, 6 per cent die later of tuberculosis of the lungs , in the second ten years, 9 per cent , of those who have passed 20 years, 7 per cent

The functional results, in spite of atrophy, shortening, ankylosis, and vicious position he considered good In all cases motion was limited Ankylosis was a frequent result of suppuration In every case the limb was in a vicious position of flexion, and usually adduction, rarely abduction—the flexion sometimes being 90 *per cent* Flexion, rather than shortening, was the cause of the functional disability. The shortening was due to three factors Atrophy of disuse, absorption of the *femoral head*, and extension of the acetabulum in an upward and backward direction due to absorption of bone Without giving any statistics of resection cases he *claims* that their results, in view of life and limb, are no better than these His qualification to his advocacy of conservatism was his desire to await modern methods of wound treatment in resection cases At the same time, Schede, of Hamburg, Helferich of Griefswald, Gussenbauer, of Prague, and von Bergmann, of Berlin, advocated conservatism as against operation

There are several points in *these* statistics which would bear careful discussion, but I must not, in my allotted time, attempt more than a brief mention of one or two His total percentage of deaths, 40 per cent is a higher mortality than is usually credited this particular form of joint tuberculosis, but this is explainable, partly, on the fact that many of his cases occurred in the time before systematic treatment by traction and immobilization was instituted The mortality of the suppurative cases, 58 per cent , is of the whole report the part that most holds attention According to it the chances of a given case's dying are more than doubled by *the* occurrence of abscess There can be no doubt in this world but that it is proper to persevere in conservative treatment in cases where there is no abscess for the ultimate chances of recovery with a useful limb are good But if abscess comes it is a symptom that the organism is not able to absorb and eliminate the detritus of the infected tissue—tissue that is potentially or actually dead and must be removed, the disease is gaining on the patient, conservative treatment is not conserving At this point of any particular case what sign or symptom will tell us the the patient's chances, whether he will, after being more or less ill, ultimately recover or whether he will die We know he has forty-two chances of recovering to fifty eight of dying, but what is this particular patient going to do? How long should conservative treatment be persevered in under these circumstances? That is, how long should the patient be left to the care and efforts of the staphylococcus to rid him of his diseased bone when the original infection, the bacillus of tuberculosis, may be extending its operations in the bone piece by piece, or migrating to and attacking other organs?

These statistics are the latest I know of that cover a considerable number of cases They have been compiled under strict stipulations as to what cases were proper ones to be included In spite of the consensus of opinions in favor of conservatism I cannot see but that the statistics themselves force us to ask these questions, questions which [illegible] himself suggested when he stated that he wished to know the results of later methods of general treatment before finally committing himself It is, then, my duty, in the present instance, to attempt to elucidate the matter

CONSERVATISM IN THE TREATMENT OF TUBERCULAR JOINT DISEASE

[illegible] at the Forty-fifth Annual [illegible] held at San Fran-[illegible]

BY JAMES E THOMPSON, M B, B S, Lond, F R C S, Eng

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The days of conservatism, to judge from a perusal of our medical journals, seem almost to have passed, for no sooner does a student gain the magical cognomen of M D than he seeks fields and pastures new where he may acquire local if not world-wide fame He seeks this, not in the direction of honest, hard, painstaking clinical work but only too often sees his chance in some major operation, requiring not alone judgment in its planning but experience in perceiving its necessity Too often in his endeavors to count his majors by the tens or hundreds he loses sight of the main object of his art, which is to save nature's organs intact as far as is compatible with health and life, and errs in his enthusiasm wilfully if not criminally in producing mutilations that cause older and more conservative surgeons to shudder in dismay Thus it is that our journals are flooded with records of hip joint amputations, heroic operations on the spine and such like, to the exclusion of records, which happily are being collected, where the limb still remains as a monument of the patient efforts of some obstinately conscientious practitioner, who, following nature's dictates and methods succeeds in saving life and limb in spite of the sneers and innuendoes of his fellow practitioners However, it is possible for us in our admiration of conservatism to let the pendulum swing too far and to lose sight of the fact that mutilation is not naturally the antithesis of conservatism, but, that between the two there lies a field where bold, active surgical measures may be of paramount benefit to the patient and absolutely indicated in the various stages of the disease It is this boundary line between conservatism and active treatment of a tubercular joint that I wish to discuss in some detail, and to make as far as possible a clear distinction between cases that require operation and those which will be decidedly the better for absolute unhindered rest

There are so many considerations that enter into a calculation of this nature and which influence one's opinion in coming to a just conclusion that it is impossible to lay down definite rules which will hold in every case Diathesis, present health, social condition particularly the power to gratify certain requirements, such as climate, food, apparatus, etc, influence so materially the conduct of cases, that each patient must be judged on his or her own special condition and environments

Given a patient in the first stages of hip-disease, a patient whose social condition is such that every luxury can be obtained, that change of climate, fresh air, conveyances, the best of food cooked in the best way, are all procurable, a patient whose common sense or the moral control of whose friends would help in carrying out instructions as to rest in bed, the use of apparatus and the like, I should have little hesitation in giving a very favorable prognosis In the great majority of such cases cure would follow and, with care in after treatment, the cure would be permanent

On the other hand, a case of this sort denied all care, all necessities and all luxuries, and, especially denied intelligent supervision and nursing would sooner or later progress from bad to worse and would end, as most of these unfortunate cases do end, in total destruction of the joint and perhaps in death from exhaustion This is the course with only too many patients seen in our crowded cities, everything is ordered but nothing procured, with disaster as the result In the face of this, can one wonder at the surgeons in crowded manufacturing centers advocating and carrying out excisions at the very earliest period, even before the joint cavity is involved, under the perfectly legitimate idea that it will only be a question of a short time before the process will go on apace and destroy the articulation?

These advocates of early excision undoubtedly go too far in their views, and often a joint which could be saved with the limited means at their disposal is sacrificed ruthlessly as a holocaust on the altar of their faith I have among my collection of hip joints one which was removed by one of my old teachers which shows two caseous foci close to the epiphysial line, the joint otherwise being healthy Knowing as I do both the operator and the squalid surroundings of most of his patients, I am fully prepared to accept this specimen as an evidence of skilled diagnosis and appropriate treatment However, I think that if the social conditions had been otherwise that it is just in such cases, viz, those where the disease commences primarily in bone that conservative treatment will show the most brilliant results

Diagnosis is everything and an error committed in the early stages may result in a disaster to the patient It has been my luck to see cases of commencing hip disease in children diagnosed as intracapsular fracture of the femur and subjected to rough passive movements under chloroform Most of these cases ended in excision, and in one a subacute osteomyelitis set in, which necessitated removal of two or three inches of the shaft and scraping out the medullary cavity So then, I would lay it down as a dictum, that where the disease can be diagnosed as confined to bone, conservative treatment should be relied on as giving the best results

This is seen to a remarkable degree in tuberculosis of the vertebræ where perforce, in the majority of cases, conservatism is the only available treatment One sees daily, numerous living examples of healed spinal caries where we have deformity but no other trouble Here the disease is primarily in bone and is surprisingly curable when allowed to have a chance Sometimes, in spite of maltreatment, it will clear up in a marvelous manner

The relation between symptomatology and conservatism is a close one, as it is only in cases where the diagnosis is made early before the joint is extensively involved, that we can hope for a good result Everything, then, hinges on an early recognition of the joint disease and this, in skilled hands, is usually an easy matter, although cases occasionally occur which for a time defy a positive opinion I would here be dogmatic and lay it down as an absolute rule, that, in cases of doubt, the patient should be treated as if the suspected joint were the seat of a veritable tuberculosis

The slightest stiffness of or unwillingness to move the joint in a patient of a tubercular diathesis should be viewed with suspicion, and these symptoms accompanied by pain should at once be a signal for absolute enforced rest We should be just as much on the alert to diagnose our joint cases at their curable stage as we are to diagnose malignant disease when it is essentially local and amenable to treatment One could cite cases innumerable where patients who had suffered from the early stages of hip disease during infancy had by careful treatment recovered full movement of the limb, and were in adult life to all appearance free from tubercular foci It is impossible to say whether the bacilli or their spores are merely latent and awaiting a suitable opportunity to blossom into life, and knowing as we do the tendency of a once actively tuberculous focus to awaken, we are very careful to advise moderation in using a joint once diseased

The cases that are the most troublesome are those where it is difficult to come to a decision as to the employment of operative procedures In other words, when should we advise rest and when operation?

The answer to this depends mainly on two considerations 1, on the joint affected, 2, on the situation and extent of the disease in the particular joint 1, considering all things the joints of the upper extremity are more amenable to conservative treatment than those of the lower, because they can be more easily rendered immovable and are less liable to be injured during locomotion, added to which the patient can obtain exercise and fresh air without risk The joints of the spine occupy a peculiar position in being almost beyond the reach of accurate surgical treatment, in the early stages at least

2 In considering this section, with due regard to what I have previously said as to the views of the advocates of early excision, I should be very much inclined to doubt the efficacy of rest and conservative measures generally in joints where the symptoms pointed to the destruction of the articular surfaces and pus formation In these cases I should feel it my duty to advise something more than the expectant treatment

The particular joint affected and the anatomic region involved would also modify my attitude towards conservatism in any given case thus in cases of tuberculosis affecting, and appearing to be confined to the synovial membrane of the knee joint, while recognizing the fact that here rest is a powerful aid towards cure, I should have less compunction than formerly in the face of brilliant results, such as I have seen from arthrectomy, in advising this procedure It is in these cases, however, that treatment by rest associated with pressure or passive congestion (Bier's treatment) has shown its best results, and consequently the greater one's experience the less dogmatic would one become in favor of or in opposition to any particular method Occasionally one may be shaken somewhat in one's power of judging as to whether operation is necessary or not A colored girl aged 7 was under my care some two years ago suffering from spinal caries affecting the fourth, fifth and sixth dorsal vertebræ associated with absolute paralysis of the lower limbs, no anesthesia and no implication of the bladder and rectum There was marked angular deformity but no evidence of pus formation The temperature varied and occasionally rose as high as 101 degrees F in the evening Absolute rest was enjoined and during six months the child was encased in a well fitting Sayre's plaster jacket but with no effect In despair, I had made up my mind to operate and the jacket was removed for that purpose but for some reason the operation was postponed for two weeks, during which time the child was allowed to roll about the ward floor To my surprise, I found she was regaining the use of her limbs, and I postponed the operation indefinitely and had the satisfaction of seeing her recover the use of the lower members entirely Had I operated, I should have attributed the improvement to the operation and not to nature's own processes

Having made up our minds to put any particular joint at rest, the method of carrying out our plans deserves some description The forms of apparatus for each joint are legion and most are good However, as a rule it is not the particular apparatus which is going to cure our case, but the individual care expended on it by the surgeon and his assistants. Wherever practicable the joint should be fixed immovably and shielded from traumatism, and usually plaster-of paris is the best material to use In the case of children afflicted with hip disease the limb is fixed firmly in a plaster case from foot to pelvis, and the same bandage is carried over the sound joint as far as the lower part of the thigh I have, however, found it impracticable to use plaster-of-paris during the hot weather of our summer and have been forced to be content with rest in bed with extension, using sand bags to steady the body and limbs The enforced rest is absolute and is kept up for one year, the patients' not even being allowed to sit upright during the early part of this time Each day the patients are wheeled out on the gallery and allowed to bask in the sun, as I find a remarkably good effect from sunlight and fresh air

Is it possible that a limb can deteriorate by rest too prolonged? The answer to this reveals some curious facts by a perusal of the rather scanty literature of the subject

Teissier[1] showed by dissection that long continued immobility in a joint might produce a series of important changes including synovial effusion, permanent shortening and thickening of the ligaments, swelling, softening and erosions of cartilage, and even true fibrous ankylosis

Volkmann reported a number of cases of transient effusion which appeared in joints which had been immobilized in the treatment of fractures and other diseases

[illegible] experimented on animals by confining their limbs in plaster of paris and found well marked degenerative changes at the points subjected to the greatest pressure Further information and researches will be found in papers published by Reyher,[4] Paget[5] and [illegible][6] and all go to prove that certain changes [illegible] in healthy joints kept at rest for [illegible] length of time Notwithstanding [illegible] effects of rest on diseased joints are [illegible] to lessen the value of this procedure

In [illegible] especially those where starting [illegible] form a prominent symptom it may [illegible] to put extension on the limb the object [illegible] the joint surface and to pre[illegible] pressure The method of appli[illegible] as it may be possible in [illegible] the symptoms which it is in[illegible] Thus in some instances of extreme [illegible] associated with shortening of the [illegible] extension be put on and the line of [illegible] horizontal, the head of the femur [illegible] into the acetabulum and more pres[illegible] to bear on the articulating surfaces, [illegible] being a lever of first kind, the power [illegible] the lower end of the femur the ful[illegible] insertion Therefore extension [illegible] at first always be applied in a direction paral[illegible] the shaft of the displaced bone, in cases of hip [illegible] parallel to the flexed femur, in knee joint dis[illegible] parallel to the flexed leg Otherwise extension [illegible] not only increases the suffering of the patient, but is [illegible] harmful Later on, when one has overcome the resistance of the contracted structures, it is possible to change the direction of the extension and gradually to bring the limb into the desired position

The value of extension depends on the power of separating the diseased surfaces from one another and so preventing undue injury by friction The relief that a patient experiences from starting pains at night is too marked to doubt the efficacy of this procedure

When we ask ourselves what are the limits of the treatment by rest, we are unable to fix any arbitrary boundary line As long as there is any evidence of inflammation present, such as pain and tenderness, and especially if there be suppuration, rest must be continued It is advisable in all cases of early tuberculosis to err on the side of too prolonged rest rather than to allow movements in a joint where there is the least chance of the disease being active But the question naturally forces itself on us, Are we to use passive motion in a previously diseased joint when, from the absence of pain and tenderness, we have reason to believe that the tubercular process is in abeyance? This is always a very difficult question to answer At times, especially where the tubercular disease is confined to the synovial membrane, massage combined with moderate passive motion may be of benefit, but I am not inclined to view this procedure with approval Yet when one compares the treatment which is advised in pulmonary tuberculosis with that usually followed out in joints, it seems at least curious that in the lungs exercise should be found beneficial and, in fact, in moderation absolutely necessary for a favorable termination, whereas, in joints one condemns exercise as deleterious Is it possible that full inflation of the lungs increases the vascular supply and helps the connective tissue cells to fight the bacilli without deleterious consequences to the intrinsic lung tissue, whereas movement in joint surfaces injures the intrinsic tissues without in any way bettering their nutrition and placing them in a more resistant attitude? I am not prepared to believe this difference, but am rather inclined to think that the processes are identical and that, if we could only interpret aright the conditions favorable to passive motion, there are many joints that would be decidedly benefited But the difficulties in the way of a correct knowledge of the pathologic condition of a joint are so great that any attempt to move a joint is attended with more risk than is justifiable

As adjuncts to rest we have such means as compression, counter-irritations and massage All these means have a limited application and considered alone a very doubtful value Compression is particularly useful in cases of tubercle confined to the synovial membrane and where we have no evidence of a central tubercular osteitis It may be applied with a rubber bandage or by strips of adhesive plaster over a cushion of cotton

Cold may be applied during the non-inflammatory stage by means of an ice bag or a Leiter's coil One should, however, guard carefully against its abuse as it may interfere with the proper circulation of the part

Heat has a more extended field of application It is best used in the form of antiseptic fomentations, medicated if thought necessary with iodin, corrosive sublimate or boric acid We may also surround the joint with a Leiter's coil and keep up a continuous stream of hot water running through the tubes

Blisters actual cautery, setons, moxas have no place in the modern treatment of the disease They only incommode the patient and have no beneficial effect on the diseased joint

Bier,[7] of Kiel, has advocated the treatment of tubercular joints by a method which he calls "*passive congestion*" The procedure, which is simple, consists in placing a broad elastic band around the limb a few inches above the diseased joint, firm enough to produce congestion but no edema, and loose enough to run no chance of rendering the limb anemic This congestion is kept up continuously and the movements of the part encouraged, the elastic band being changed occasionally to prevent chafing and abrasion The congestion is confined to the immediate neighborhood of the joint by carefully bandaging the distal portion of the limb

The idea was suggested to him by an old dictum of Rokitansky's, that all cyanosis is incompatible with tuberculosis, a remark which however true in its main features is not absolute, for one occasionally sees phthisis associated with congenital heart disease

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[1] Gazette Médicale, Sept 25, Oct 2, 1841
[2] Berlin Klin Wochenschrift, Nos 30, 31, 1870
[3] Arch für klin Chir, Bd XII
[4] Zeit für Chirurg, Bd III
[5] Clinical Lectures, p 97
[6] Transac Patholog Society, Vol XXV, p 212

[7] Centralblatt für Chirurgie, No 32, s 57, 1892 also Behandlung Tuberk der Gliedmassen mit stauungshyperæmie, 1893

I have had no experience in this method, but will quote the conclusions arrived at by Miller[8] who has put this method to a fair test He says that his experience has not equalled that of Bier, as most of his cases were afterwards treated by other means and could not under the rules of the hospital be allowed to remain a sufficient length of time in the wards In summing up he comes to the conclusion that the new method can not in the majority of cases take the place of amputation, excision, scraping nor of immobilization, but that combined with immobilization it is undoubtedly an aid in the treatment of these affections

A limb with a tubercular joint has smaller arteries and is in consequence inadequately supplied with nutriment May it not be that the diseased part is on account of its malnutrition unfavorably situated as regards its contest with the tubercle bacilli, and that the congestion of the neighborhood assists in the destruction of the bacilli and the throwing off of the disease by bringing more blood and serum (pabulum) to the fighting tissue?

[8] Edinb Medical Journal, February 1894 p 702

THE OPERATIVE TREATMENT OF TUBERCULOSIS OF JOINTS—(EXCISION)

Read in the Section on Surgery and Anatomy at the Forty fifth Annual Meeting of the American Medical Association held at San Francisco June 5-8 1894

BY ROBERT W LOVETT, M D
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The question of the operative treatment of joint disease should involve the discussion of several procedures, excision, arthrectomy, incision and ignipuncture The limits of this paper, however, compel the writer to confine his attention to the consideration of the place of excision in the treatment of tuberculous joint disease On this subject there is a wide range of opinion in the surgical world Some surgeons advocate early excision as a routine treatment in tuberculous joint disease, others would never excise at all, because they consider the operation as rarely necessary, and as dangerous, and a hird faction believes that excision is to be kept in he background until conservative measures (i e, mechanical treatment) have had a faithful trial

Mechanical treatment is a term which has been ften applied to the loosest and most incomplete im-tations of proper conservative measures Conse-uently, when one attempts to define the sphere of perative measures, contrasted with mechanical ones in joint tuberculosis, it must be understood that by mechanical treatment is meant here the intelligent nd systematic application of suitable apparatus by urgeons of special training, not the reference of a atient to an instrument-maker with the order for a hip splint" This is not the mechanical treatment hich yields good functional results

There are two dangers from excision of joints 1, ss of life, 2, generalization of the tuberculosis

1 Excision of even the larger joints is not an peration which is accredited with a high death rate f late years the mortality has become much less, ut the immediate mortality is of little significance mpared with the remote, of which we know very little, for few figures deal with the latter Immediate results in these cases show very little, because relapse and generalization of tuberculosis occur later, and are the really significant factors Just as any statistics of operation for cancer of the breast, which only dealt with the three months after operation, would be of little or no value in determining the value of the operation as a cure of cancer As an example of the very favorable aspect of operative treatment considered (as usually is the case) in the light of immediate results, the following figures are of interest, taking a group of the latest results in excision of the knee for instance

Lucke[1] reports 101 excisions of the knee with 85 per cent of good results, Lucas Championniere[2] 44 excisions without a death, Thompson[3] reports 50 knee excisions without a death in 287 cases in adults, reported by Schulter[4] 12 5 per cent died, and in 274 cases under 20, 10 9 per cent died In 129 excisions, reported by Bothe, there was no death[5], Zeuge-Mauteuffell[6] had 11 per cent of deaths in 55 cases done at the Dorpat clinic

In Lucke's cases there were 60 per cent of cures in a series of 48 excisions in children, reported by Angerer, below the age of puberty, there were 70 per cent of total recovery, in the series of 561 cases, reported by Schulter, which have been already alluded to, 63 8 per cent were cured In the last 61 operations of the 131 reported by Boeckel[8], union without fistulæ occurred in 82 per cent In the fifty-five cases operated on at the Dorpat clinic, union by first intention occurred in 71 per cent As was said by Verneuil, at the Congress for Tuberculosis "We have reason to be optimistic when it concerns immediate results"

Turning to remote results there are fewer figures Korff[9] reports the final results in 104 cases of general resections, with a mortality of 37 5 per cent, Boeckel relates 127 resections of joints in general There were sixteen deaths soon after operation (nine from tuberculosis), and fourteen late deaths (eleven from tuberculosis), a mortality of 24 per cent at least

The class of cases from which these statistics are drawn is not one from which the remote results can be well estimated, so easily do they escape from observation In the more inaccessible joints, such as the hip, the results are not as favorable as to recovery rates In 2,461 cases of hip excision, collected by G A Wright[10], the mortality was 34 per cent

2 Generalization of tuberculosis This danger demands serious consideration, because it is a danger which the improvement of antisepsis has not tended to diminish Wartmann[11] analyzed 837 resections and found that 10 per cent of all deaths were the result of rapid miliary tuberculosis And Mr Barker, a radical advocate of excision, stated in a lecture before the Royal College of Surgeons in 1888, that in 10 per cent of all deaths following excision, "rapid miliary tuberculosis came on in such a way as to suggest strongly, if not to prove, that the surgical interference was the cause of the generalization

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[1] Deutsch z für Chir., xxix, H 4
[2] La Semaine Medicale Aug 6 1891
[3] Dublin Journal Med Science December 1889
[4] Deutsch z für Chir., xxx, p 285
[5] Schmidt s Jahrbuch, June 1891
[6] Deutsch z für Chir., xxix p 113
[7] Wein Med Wochensch June 21 1891
[8] La Semaine Medicale April 12 1892
[9] Deutsch z für Chir xxii p 149
[10] Hip Disease in Childhood, London 1887
[11] Deutsch z für Chir., xxiv 435

nent are these When in spite of good care, joint swelling increases, dense induration comes on with discharging sinuses, the granulations become flabby and inactive, and when, most important of all, the general condition of the child has begun to fail, the time for operation has manifestly come Certain painful cases, which are rare, demand excision as soon as it has been demonstrated that mechanical measures have failed to control the pain

The chief index, however, in the care of children is failure of the general condition along with a bad condition of the joint Either alone is incomplete This states only the view of those surgeons who are familiar with and believe in mechanical treatment The loud advocates of operation are, as a rule, those surgeons at home and abroad who know little or nothing of the best conservative measures In adults, tuberculous joint disease is from the beginning a more serious matter than in children The process is more acute, it is not so amenable to mechanical measures, the systemic infection is more, and in cases of even moderate severity, excision is, as a rule, the best treatment The results of mechanical treatment are not satisfactory except in mild cases, nor should mechanical treatment be persisted in for more than a brief time if progress is unfavorable Nor are the results of excision so successful as in children, although the perfection of operative methods have tended to improve the death rate and the results

The writer has avoided burdening this paper with masses of comparative statistics bearing upon the results to be obtained by mechanical and operative measures A list of recent and important references is appended

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SECTION ON SURGERY AND ANATOMY

SECOND DAY, JUNE 6

Papers on "Early Symptoms and Diagnosis of Tubercular Joint Disease" were read by DRS EMMET RIXFORD, San Francisco and A J STEELE St Louis, Mo A paper by DR A B JUDSON New York on the same subject, owing to his absence, was read by the Secretary

Conservative Treatment of Tubercular Joints," papers by DRS R H SAYRE New York and HARRY M SHERMAN, San Francisco were read by the authors, and one by DR JAMES L THOMPSON Galveston, Texas, in the absence of its author, was read by the Secretary

DR ROBERT W LOVETT S paper on "Operative Treatment of Tubercular Joints" was also read by the Secretary, its author being absent

'Treatment of Tubercular Joints by Injections of Iodoform," was a paper presented and read by DR STANLEY STILLMAN, San Francisco

"Treatment of Tubercular Joints by Injections of Corrosive Sublimate," by DR R H PLUMMER, San Francisco, was read by title and referred for publication, as was also a paper by DR A E HOADLEY, Chicago, on "Common Errors in Prescribing and Applying Mechanical Apparatus"

DISCUSSION

DR MEYER, of San Francisco—I take the pleasure of introducing a patient with tuberculosis of the elbow joint, treated by combining operative (incision and scraping) with Bier's method of constriction The wound apparently healed quicker, while it has quite dangerous symptoms as to discoloration, it is noticed that a line of demarkation forms, a slough is thrown off, and the wound apparently heals quicker than by other treatment This case is only put on exhibition as a suggestion to those members present who have an opportunity to try this new method and report at subsequent sittings, or at some future association as to the value of this treatment While with the regular incision for resection of the elbow joint there may be considerable of the humerus scraped out, the wound here cicatrized in less time than usual, taking in this case only five weeks And for that reason I would recommend to surgeons, who have an opportunity, to try and see whether by this method we make more headway than by iodoform and other treatment with operative interference

DR H M SHERMAN, San Francisco (introducing three patients)—I wanted to give as nearly as I could an example of a later result of hip resection, to show what the work is This child has been operated on now, I think it is something like four years For the last two years she has used no support in the way of a crutch or a stick, for her limp, the shortening is accurately corrected by the shoe Her limp is the lateral list, the unavoidable mechanical result of removal of the neck of the femur She has perfect support and a very good functional result in being able to move the limb around in almost any way she wishes The lateral list is practically the only thing she has and there is very little of that In making a table of cases, she would be put down as a child having a very slight limp, and technically it is not so—her limp is a list

It is seven years since this boy's hip was removed He had also at that time a dorsal spinal tuberculosis, and was wearing a leather appendage, and a little apparatus to carry his head His limp is less than the other, but that is because he can not make the lateral sway, his spine is too rigid

I think it is nearly three years since this young gentleman's hip was removed He ordinarily uses simply this cane The shortening is made up by one of those patent boots, in the position of extension, so that nothing shows Now that is a bad limp, but this gentleman was older than the children when he was operated upon And with his stick on the other side that limp becomes insignificant His hip had become ankylosed so that there was abscess, the bone was really in a very bad condition, and he elected himself to have the operation done so that he could go hunting, and riding on horseback, which he could not do very well with an ankylosed hip He spends a good part of his time on horseback, and does everything that anybody else could want to do

As regards what Dr Rixford said about the diagnosis, and postponing the beginning of treatment until he is sure of his diagnosis, I must object to that If a child comes to me and I suspect there is tuberculosis of the bone, I begin treatment at once Afterward if I find out that I was mistaken I have done no harm, and am very glad, indeed, under the circumstances to be mistaken and to have made an error in diagnosis, but I do not want to wait, to be absolutely sure that there is tuberculosis of the bone, before beginning my treatment, and let the disease get ahead of me I took a splint off of a child the other day for hip joint disease, where she had worn it only a year, and where she had apparently no remnant of the disease at all, and I told the mother I doubted very much if the child had had tuberculosis of the bone, she had gotten well too quick

Then as regards the iodoform injections In the first place, it is as well perhaps for us to remember that it is not an emulsion, it is a suspension of iodoform in whatever vehicle you choose, but it is not an emulsion I am disappointed in the treatment I have had a series of cases at the Children's Hospital which have been watched very carefully, the injections have been made, and I have failed to see that they in any way affected the progress of the cases In no instance did it do the child any harm, but I do not think that it has made the recovery any more rapid, or that there has been any effect from it

A tissue that has been infected by the bacillus tuberculosis is destroyed, it has to be removed, and the injection of iodoform will not postpone the removal of it Nature will do that in those cases that do not suppurate, by the absorption of the infectious tissues and their replacement by cicatrix When suppuration takes place the staphylococcus thrives on the infected tissue, and it is broken down and comes out through the sinus I do not see how, when once it is firmly established, the injection of iodoform, even into the tissue itself can in any way facilitate that process It might perhaps kill the bacillus tuberculosis, but then its effects are still there in the shape of tissue that was infected, and that either potentially or actually is dead tissue

Dr J [illegible] Iowa—The general tendency of Dr [illegible]'s paper was in favor of conservative treatment in the treatment of hospital cases, and where the patients can be kept constantly under the observation of the attending surgeon, I grant that the statistics are better under that class of treatment than under the operative In private practice and in consultation work, both in your cities and country towns such as many surgeons are engaged in, I [illegible] that the difficulties of conservative treatment are in many cases insurmountable, and I am of the opinion that in the great majority of cases under these circumstances the operative system will give better results and will save long suffering to the patient Operative treatment, if effectual at all, offers rapid amelioration and cure of the disease Tedious treatment by conservative treatment, by injection of different agents [illegible] and by the application of mechanical apparatus is in a great majority of cases tedious, and in the class of cases to which I refer extremely unsatisfactory in my estimation

Dr J [illegible], Washington—The gentleman has said exactly what I wished to say I will simply add one word to what he has already said, that the ability of the average general surgeon scattered about the country in isolated places, to treat the conservative methods of treatment must certainly be limited always I care not how thorough the discipline the person has been subjected to if he is placed where he will see one of these cases once a year or twice a year, or half a dozen times in a lifetime, his ability to handle them from the conservative standpoint must be minimized, to say the least Hence I say that the dealing with this class of cases must be looked at from the standpoint of the operation then under those circumstances, and if dealt with from that standpoint then I say that the necessity for early interference is all important It is little less than criminal, it is cruel to watch the progress of a case for months and months, and perhaps for years with the promise that at some time you will interfere to give possible relief by operation I believe that an early operation in these cases is the resort of such persons under such circumstances I believe that can not be emphasized too strongly

Dr Kurtz California—It is too late to enter into any close discussion on this subject I simply want to direct your attention to two points In the first place it seemed to me that there was a discrepancy in the papers in regard to early diagnosis Perhaps it is trifling, but I think it is of considerable importance The first reader, I believe Dr Judson, of New York, pointed out that we have, as a first symptom, pain I think that is certainly incorrect, according to my experience and according to my study It is not the pain that first appears, but it is lameness, and that should be well remembered, for it is of very great importance A child will drag his leg, it will slightly limp and that limp of the patient with hip disease is very characteristic, he drags the whole leg, not *one* part as in other diseases, and if you examine that, you will find neither pain nor limited motion, but you have that peculiar dragging limp, probably for a month or two months before the subsequent symptoms, the pain sets in The same reader also made the remark that we should be perfectly satisfied in preliminary diagnosis before instituting treatment Really, if we want to wait for the pain that he points out as being the first symptom, I think it will be too late We should rather make a mistake on the safe side and put our patient in bed with his lameness for a week, or a month, or six weeks, or even more, and see if we can not overcome that condition If we put a child unnecessarily in bed for a month or two months what does it matter? He will not suffer from it So I say, begin the treatment as soon as you notice his dragging limp Dr Sayre in his very able paper seemed to lay great emphasis on the absolute rest cure in cases of spinal disease, he lays great stress, I say, on the absolute rest cure This would be the ideal means of treatment, to place your patient on a hard bed, as he says, or board, and keep him there, with or without a bed I say if we once put a child on a hard bed, with extension, we need no apparatus at all We all agree that it takes from two to four or six years to cure any of those cases of Pott's disease, or coccyxitis, or any tubercular disease of the joint, with the exception of the smaller joint So I say it is rather a risky thing to lay a child on his back that long Give that child a comfortable splint, give him a Bostwick splint, or some modification of it, and you will have fully as good results That is my experience I prefer a living slightly deformed child to a straight dead one

Dr Lyon, Conn—It seems to me that this case, (referring to the case exhibited by Dr Meyer) is proper to illustrate the subject before us, and I wish to make one remark in reference to it He tells me that he was injured eleven years ago by the crushing of this elbow joint He has now a well-nigh useless arm, but he has a valuable forearm and hand if he could make it available Now if that elbow joint had been taken out, two to four inches of it, he would have had, not a useful forearm and hand, but he would have had a useful arm That interference with the nutrition of the arm made it a proper site for the infection of other diseases He has now a wasted arm, but if that had been taken out, I venture to say, from two to four inches, and allowed the bone to come together, this lad would have had a useful forearm and hand

Dr Meyer—I wish to say that this case was under my treatment only since June, 1890, that I had nothing to do with the primary injury, as eleven years ago I never thought of the study of medicine even I only take this case to illustrate what can be done by combining the treatment by constriction in operative cases, and showing what can be done in a comparatively short time, in six weeks after the operation was closed He was very much emaciated then, but is now better in that respect, and I intend to make a typical resection of the elbow joint, trying to get a movable elbow Eleven years ago I did not have this case

Dr Thomas, Pa—With regard to the statement that pain is not the first symptom of disease of the joint, I think that the child who is too small to let us know the fact that he has pain will limp, that very limp is an indubitable sign of pain, it can't be otherwise

Dr George F Shiels, San Francisco—Apropos of this point concerning pain, I think that this word, pain, is merely a word to express an idea. No matter what we call it, a feeling of weakness in the bone, carrying to the individual the idea that it is not strong enough to support the body, will cause him to limp to save that bone If the individual feels that there is a weakness in that joint and that he must protect it in such a way as to relieve it, and thereby causing a limp, I think it is a very small matter to argue about whether it is a limp or a pain It is certainly something by which the patient is made aware of the fact there is a weakness in that joint and that it has to be protected by walking in a particular manner, which produces what we call a limp

Dr R H Sayre, New York—There are one or two points that have been made to day which I would like to dwell on I fully indorse the remarks of Dr Shiels, and also in that connection I would like to say that when Dr Rixford says he does not get pain in these cases it is probably because he does not cause sufficient pain in the child for the child to cry out and complain The truth, however, is that it has a little spasm of the muscles, that there is a twitching of these muscles under his hand, as he moves his hand up and down, however the child may say it does not hurt You will find sometimes these children say it does not hurt, while they cry Very often they are too little to understand enough of your language to express their feelings properly, and you must be guided by their faces and see if they express sensitiveness in the eyes and mouth, then they have pain, although they very likely tell us they have no pain And Dr Shiels is perfectly right when he says that the involuntary protection to the joint which the child gives by its limp, by its little muscular spasm is a symptom that it has pain I think that to avoid the beginning of treatment till the child has a marked limp, has deformity, a hump in its back which makes you positive that your diagnosis is correct, is a distinct mistake If you wait until that time you will never get an absolutely good result in your treatment You have passed the time You might as well expect a man who has a little hacking cough in the morning, who has slightly elevated temperature, who gives a little bronchial breathing in the upper part of his lung, to wait until he has a large hemorrhage in his pulmonary cavity and expect to cure him of his consumption, as to wait until this child has a hump in his back and a limp, rather than confine him for a few weeks at the beginning, for fear it may prove ultimately to have been unnecessary Sometimes pain is due to an involvement of the cartilage In many cases we have a marked presence of the disease before there is acute pain Unless there is an abscess forming or the cartilage is involved, very often there is a distinct disease before there is alarming pain But a little pain is what we want to find We want to be able to take this thing in its incipiency

In regard to these statistics which Dr Sherman read, that no case recovers which is over 40 years of age, that I know to be a distinct mistake I have seen cases of tuberculosis over 40 years of age which have recovered I know of a number of cases in my father's practice, and several in my own, where the patients were over 40 at the time of their first symptoms and they have gotten well, or, at any rate, they have lived in a comparatively good state of health, well to all appearances for varying periods until they died of old age later on

The mechanical list of which the Doctor speaks as a necessary consequence of removal of the head of the femur, I do *not think* is always present It depends upon how much bone is regenerated after the removal of the head and neck of the femur It does not always follow because you have removed a couple of inches of bone that you have a couple of inches of shortening If the cavity is packed and the periosteum left open, there is very often a large amount of bone regenerated *and it may* be sufficient in character and sufficient in size to prevent the limp I think the cases Dr Sherman showed here are remarkably good, but I think it is not always a fact that we have a limp after the removal of the neck of the femur I have seen one or two cases where that limp was not present, and where there was a very little amount of shortening, *although* extensive bone removal had been practiced

In regard to the gentleman who remarked that he preferred a living deformed child, crooked, to a straight dead one, I would also, but I do not think that to confine a child to a comfortable cuirass or keeping it in bed for even two years, or more is going to be necessarily followed by death We see that the Indians carried their children around on a board in the way I have suggested when they are small, and there are a great many Indians in the world yet And the fact that a child has an inflamed spine, and is six months or a year old, I think is a very good indication for putting that child in bed, in a little *portable* bed, and keeping it at rest The point I made was that a little child who had an insufficient pelvis on which to get a basis of support, to put his spine at rest ought to be treated *in* a recumbent position If they are big enough to run around, all right But I see lots of children fastened up in apparatus which is simply hung on their shoulders, and they have to lug around that weight in addition to their body and bones If these children were put in bed they could be transported by their parents, and when they get better they can be put in apparatus

One gentleman has said that in country practice it is *not* practicable to practice conservative surgery The object I had *in* my paper was to show that apparatus and the instrument maker were not necessary always to the practice of conservative surgery in treatment of joint diseases If you should put the joint at rest by your own intelligence and the work of a blacksmith, carrying out the principle which I treat and lay down in my paper, by *such* means as you may have in *your* bureau, plaster-of paris and so on and even resort to the blacksmith's shop for apparatus, I think that you can in any part of the country get a good result in these cases, if you will attend to the diagnosis and take the abscess before it has made vast strides There is no doubt that in our large centers we have a great many opportunities which the men *in* the country towns do not have but to say that these cases must be subjected to early treatment with the knife because they have tuberculosis is in my judgment a great mistake

The papers of Dr James T Jelks of Hot Springs, Arkansas, on "The Causation and Prevention of Hernia," of Dr L Emory Lanphear, Kansas City, Mo of Dr C M Fenn San Diego, Cal on the 'Management of Reducible Hernia', of Dr James D Eagleson Seattle, Wash, on 'The Treatment of Irreducible Hernia," were read by title and referred for publication

REDUCIBLE HERNIA

Read in the Section on Surgery and Anatomy at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco June 5-8, 1894

BY C M FENN M D
SAN DIEGO CAL.

In response to the courteous invitation of our Chairman, I have recently been endeavoring to recall the number of times the errant gut has yielded to my manipulations, and the *modus in quo* The record enumerates thirty-five reductions since 1865, most of them implicating the inguinal region, and apparently confirming the statement that 80 per cent of hernias affect that site It appears further, in comparison with Dr Dallas' estimate of 3,500,000 cases of hernia in this country alone, that the writer has measurably exceeded the ratio to each practicing physician So large a number of successful replacements, some of them following the failure of other measures, may seem to warrant a brief consideration of the method of procedure, and as I may not rightfully invade the domain of those who have been delegated to speak to you of the etiology, pathology and surgery of ruptures, my remarks will be confined to the technique of reducible hernia Not a few of the intestinal aberrations noted, occurred in the person of the same individual who, though for more than forty years a victim to those serious types of hernia known as left, oblique, incomplete, inguinal and right scrotal, attained the *ripe* old age of 79 years. We shall refer to his interesting case further on

At the inception of this discussion, I venture the assertion that hernias are not merely mechanical displacements of the intestine, but often simulate neuroses, which strangely enough are not limited to the ruptured patient The latter, if a novice in hernial accidents, or if his unaided efforts have failed to placate his ancient enemy, is frequently overwhelmed with fear of strangulation and impending death His dyspeptic, flatulent and neurotic habit, added to the emotional excitement of such occasions, causes a rapid accumulation of flatus, which in local excess arrests normal peristalsis, and with the concomitant paralysis of the gut, presents one of the obstacles to its restoration Hence, also, constipation due to paralyzing effects upon the visceral nerves This, you are aware, may often follow incarceration of the omentum alone, of the appendix, or of a small knuckle of the bowel, and in the entire absence of any contraction of caliber or impediment within the canal Furthermore, as showing the assistance during taxis to be derived from intestinal motion, to which we shall refer further on, a recent writer[1] suggests utilizing the cough, which sometimes produces the rupture—an application possibly of the Latin aphorism, "*Capilla ejusdem canis*"—the hair of the dog cures the bite The enterocele, also, is often typical of the condition within the abdominal cavity, becoming "smaller after fasting and the use of purgatives, and larger when the tube is distended with food, gas or fecal matter," and hence presents one of the indications for treatment

And how shall I describe the anomalous symptoms of the medical attendant who, at the outset of his career, if not always, seems to approach such cases with illy disguised trepidation, his brain filled with phantasms of adherent and necrotic tissues, of abnormal epigastric arteries, and other pathologic phe-

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[1] Geo Wherry F.R C S, England

TABLE 1. T/S RESPONSES OF HYPOPHYSECTOMIZED RATS TO GRADED DOSES OF TSH OR TSH CONTAINING TUMOR EXTRACT

Experiment No.	I ▲*	II ▼*	III ×*	IV	V	VI	VII	VIII	IX △*	X ×*
Substance given	TSH alone						TSH+ thyroxin	TSH in divided doses	TSH+PTU	Tumor extract
Dose mg.										
0	5.2± .7† (5)‡	7.6±1.5 (4)	—	6.7±1.4 (4)	—	—	—	3.9± .6 (5)	6.5± 1.5 (4)	—
.005	—	9.1±1.2 (4)	—	—	—	—	—	—	—	—
.01	—	6.7±1.5 (5)	—	—	—	—	—	—	9.3± 2.6 (3)	—
.07	—	—	—	—	—	—	—	4.0± .3 (8)	—	—
.1	—	8.7±1.1 (4)	—	—	—	—	—	—	21.1± 4.5 (4)	—
.2	13.9±2.4 (5)	—	8.4±1.2 (4)	—	—	—	—	—	—	—
.3	—	—	—	—	—	—	—	9.4± .7 (6)	—	—
.7	—	—	—	—	—	—	—	11.8±2.1 (8)	—	—
1.0	—	30.4±7.8 (5)	—	—	—	—	—	—	105.6±13.2 (9)	—
1.5	—	—	—	—	—	—	—	—	—	42.4±8.0 (4)
2.0	38.6±5.6 (6)	—	34.3±3.5 (5)	—	—	—	—	—	—	—
3.0	—	—	—	—	—	—	—	31.4±4.1 (5)	—	—
7.0	—	—	—	—	—	—	—	4.78±2.8 (8)	—	—
10.0	—	55.5±8.6 (5)	—	—	—	—	—	—	—	—
15.0	—	—	—	—	—	—	—	48.3±6.1 (6)	—	72.4±8.0 (4)
20.0	62.9±6.0 (6)	—	57.5±6.6 (5)	—	—	76.5±9.3 (8)	85.2±8.7 (9)	—	—	—
40.0	—	—	—	—	87.9±6.4 (5)	—	—	—	—	—
45.0	—	—	—	—	—	—	—	44.2±5.0 (6)	—	—
50.0	—	—	—	58.8±5.8 (7)	—	—	—	—	—	—
100.0	65.1±6.6 (5)	—	—	—	—	—	—	—	—	—
150.0	—	—	—	—	—	—	—	—	—	85.4±8.3 (5)

* Data of the various experiments included in this table are represented by these marks in Fig. 4.
† Mean±standard error.
‡ Number of rats in group.

companied by injections of 10 mg. of PTU in solution and an equal amount in suspension form. In experiment X the 34 hour response of the T/S to single injections of various doses of saline extracts prepared from TSH-containing transplanted mouse pituitary tumors[4] (Furth and Burnett, 1951, Furth *et al.*, 1952 a, b) was examined and compared with a dose-response curve simultaneously determined for the purified TSH preparation (experiment III). Since Ghosh *et al.* (1951) had found that a single dose of TSH was less effective in elevating the thyroid I^{131} uptake of hypophysectomized rats than the same amount of hormone given in divided doses, experiment VIII was carried out. Rats hypophysectomized 9 days previously were given 5 equal doses of TSH 12 hours apart and the T/S *determined 12 hours after the lasti njection. Table 1 shows the total* amount of TSH given per rat in each of the groups of this experiment.

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[4] We are greatly indebted to Dr. Jacob Furth, Oak Ridge National Laboratory, for the tumor bearing mice. The neoplasms used in this study were transplants of pituitary tumors, which had arisen after radiothyroidectomy, growing in radiothyroidectomized recipients. Extracts were prepared by homogenizing tumor tissue in saline. The hypophysectomized rats were injected with appropriate dilutions of the supernatant. The doses listed in Table 1 refer to the wet weight of tumor tissue extracted and administered per rat.

Figure 4 is a graphic presentation of the log dose-T/S response curves obtained for TSH (showing a composite curve constructed on the basis of experiments I and II and a curve based on experiment III), for PTU and TSH (experiment IX) and for the tumor extract (experiment X).

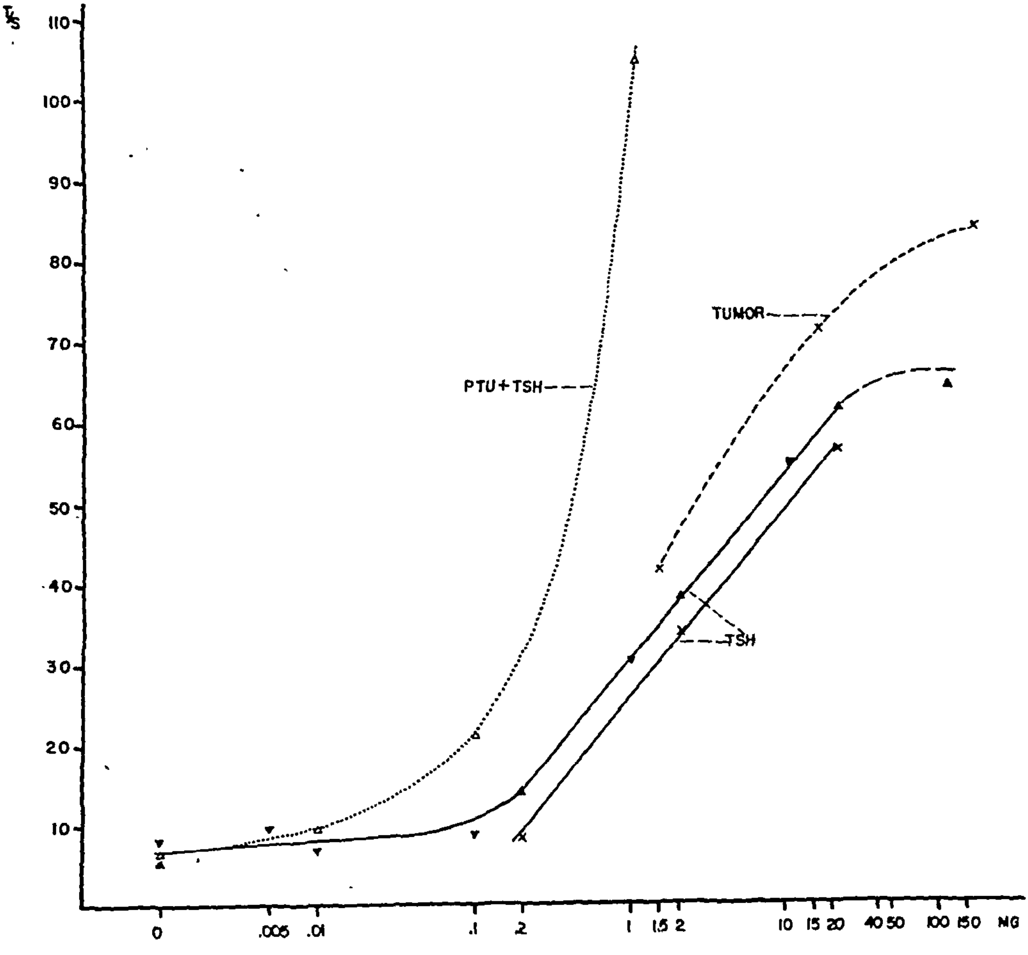

FIG. 4. Log dose—T/S response curves of hypophysectomized rats receiving TSH alone, PTU plus TSH, and TSH containing tumor extract. For further explanation consult Table 1 and text.

The quantitative data presented in Table 1 and Figure 4 can be analyzed as follows. a) The log dose TSH-T/S response curve of hypophysectomized rats appears to follow a sigmoid pattern, with the rectilinear portion lying between doses of .2 and 20 mg. Curves obtained at different times may be superimposable (experiments I and II) or at least parallel (experiment III). The great variability of responses to high doses (cf. experiments I, IV, V and VI) makes the position of the plateau shown in Figure 4 questionable. The rectilinear log dose-response relationship over a wide dosage range makes the T/S changes of hypophysectomized rats a potentially useful assay for TSH. However, the response is not sufficiently sensitive and the λ computed for the .2–20 mg. portion of the curve based on experiments I and II proved to be .489, which bespeaks relatively poor precision. b) The assay of extracts of the transplanted mouse pituitary tumors confirmed the high TSH content of these neoplasms. There appeared to be a greater concentration of extractable TSH activity in the examined sample of tumor tissue, even when expressed on a wet weight basis, than in the purified TSH preparation (cf. experiments X and III). c) One hundred μg. of thyroxin, when given together with 20 mg. of TSH, failed to have any significant effect on the T/S response to the latter (cf. experiments VI and VII. $P = .7$). d) Twenty mg. of PTU, given concurrently with single doses of TSH, significantly changed the curve of the T/S response to the hormone by increasing the sensitivity and greatly augmenting the slope for the .1 to 1 mg. dose range (experiment IX). Further studies will be necessary in order to decide whether the T/S changes in hypophysectomized rats given PTU and TSH might furnish a convenient objective method with sufficient sensitivity and precision for a practicable assay of the latter substance. e) TSH administered in divided doses was no more effective in raising the T/S than were single doses (experiments I, II, III vs. VIII). Similarly, when 10 daily injections of 1 mg. of TSH were given to 13 rats, starting 8 days after hypophysectomy, the T/S was found to be 32.7 ± 2.7[5] on the day after the last injection, which is in the same order of magnitude as the 34 hour response to a single 1 mg. dose of TSH. If there were a summation of the effects of TSH on the T/S, higher T/S values than the ones actually observed would be expected on the basis of the time curve of the response (Fig. 2) after repeated injections of the hormone.

c) *Influence of thyroxin and of PTU on the T/S response to TSH in chronic experiments*

Ingbar and Roberts (1952) stated that the effect of exogenous TSH on the T/S of hypophysectomized rats is partly counteracted if thyroxin is conjointly administered. In order to re-examine this question, we divided

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[5] Mean ± standard error.

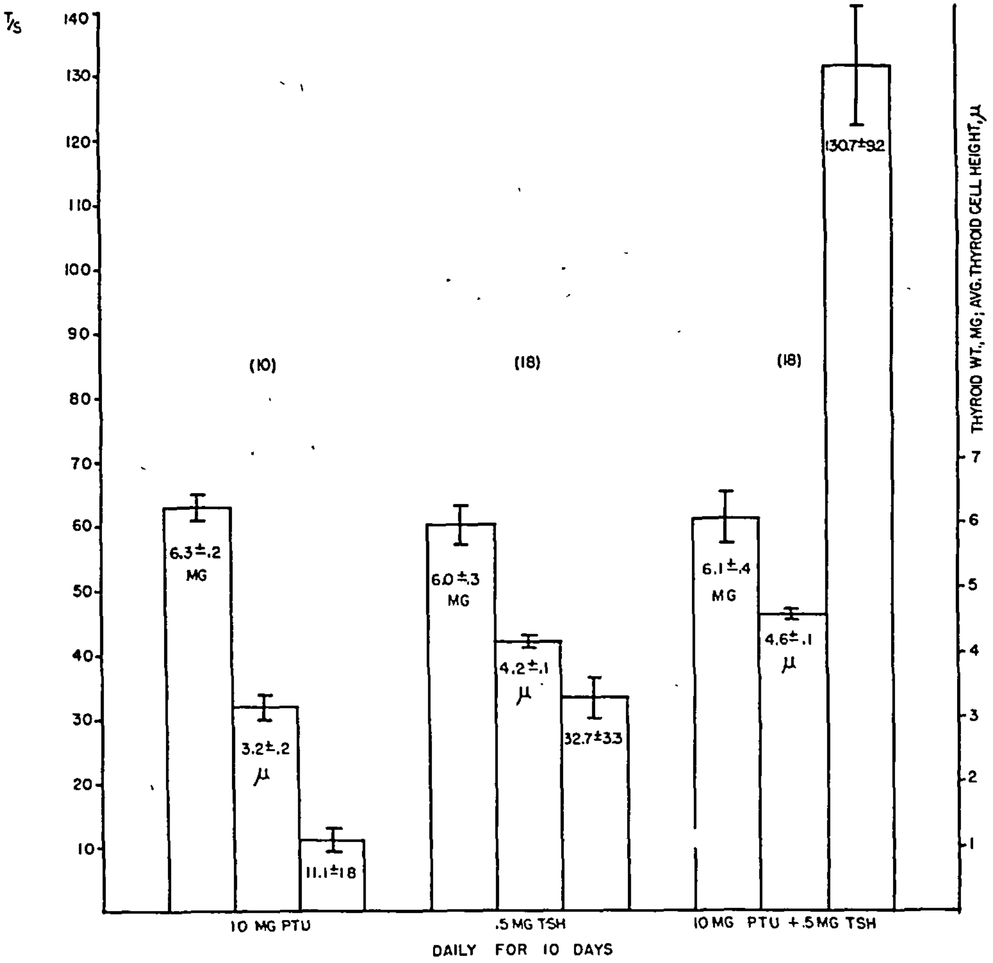

FIG. 5. Effects of PTU, TSH, and PTU plus TSH on thyroid weights, cell heights and T/S in hypophysectomized rats. Vertical bars represent mean values, vertical lines—standard errors. Figures in parentheses indicate number of rats in group, figures within bars—mean values ± standard errors.

16 hypophysectomized rats into 2 groups 8 days after the operation. Seven of the rats received 5 mg. of TSH and 1 mg. of cortisone daily for 5 days and 9 rats 20 μg. of thyroxin per day in addition. Both groups were sacrificed 24 hours after the last injection. The T/S of the former group was 36.7 ± 2.7,[5] that of the latter 39.3 ± 3.8. The difference is not significant (P = .6). A depressing effect of thyroxin on the T/S response to TSH could thus not be demonstrated under the conditions of this experiment.

The results of the last experiment of this study are summarized in Figure 5. Three groups of hypophysectomized rats were placed on the following regimens for 10 days, starting 8 days postoperatively: group 1 was injected daily with 10 mg. of PTU in suspension form, group 2 received 0.5 mg. of TSH per day, and group 3 a combination of these two treatments.[6] The day after the last injection the rats were sacrificed and thyroid weights, mean acinar cell heights and T/S ratios determined. As Figure 5 demonstrates, there was no difference in thyroid weights of the 3 groups. The mean cell height was significantly higher in the TSH group than in the PTU-treated rats, and the rise in cell height brought about by the combined treatment with PTU and TSH was slightly greater than that due to TSH alone. Owing to the low standard error the difference between the cell heights of the TSH and the PTU+TSH groups was statistically significant ($P < .01$). It should be emphasized, however, that even the most stimulated thyroids in this experiment had lower than cuboidal follicular epithelium and contained large amounts of predominantly acidophilic colloid. The T/S of the rats treated with PTU only was distinctly higher than in our untreated hypophysectomized rats but less than half that of normal rats. TSH alone raised the T/S to values slightly above the normal range. TSH and PTU together had a dramatic effect, inasmuch as they caused the T/S to soar to values more than five times as high as that of intact rats. A T/S well within the "goitrous" range was thus encountered in morphologically poorly stimulated thyroids, which is a dichotomy between the histological picture and the iodide trapping function of the thyroid similar to that described by Greer (1951) in PTU-treated rats with hypothalamic lesions.

DISCUSSION

The drop of the thyroid:serum iodide concentration ratio (T/S) after hypophysectomy and its elevation to normal or higher levels by exogenous TSH is in agreement with the concept that the iodide trapping ability of the thyroid is under the influence of TSH. The finding that prolonged administration of thyroxin depresses the T/S more than hypophysectomy does confirm similar observations reported in abstract form by Ingbar and Roberts (1952). The drop in the T/S of hypophysectomized rats effected by thyroxin appears to indicate that this hormone has a direct action on the thyroid in addition to its generally accepted depressing effect on the formation of TSH by the pituitary. Cortell and Rawson (1944), studying the four hour I^{131} uptake by the thyroid in intact and hypophysectomized rats, were unable to demonstrate a depression of this function by thyroxin in the latter group. Measurement of T/S is apparently a method better suited to the study of direct effects of thyroxin on the thyroid.

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[6] The two substances were injected in different sites.

An explanation of our failure to confirm the report of Ingbar and Roberts (1952), according to which thyroxin interferes with the reaction of the T/S of hypophysectomized rats to exogenous TSH, must await a more detailed publication of their results.

The effect of PTU on the T/S is of great interest. Although Greer (1949) had found T/S values comparable to those of our untreated hypophysectomized rats in a group of rats deprived of their pituitaries and fed PTU, our results (cf. Figs. 1 and 5) seem to suggest that PTU might have a slight effect on T/S even in the absence of the anterior pituitary lobe. This question requires further investigation. The modifying effect of PTU on the curve of the T/S response to single doses of TSH is striking (Fig. 4) and may provide the basis for an objective TSH assay comparable in sensitivity and precision to those of Ghosh *et al.* (1951). Although in our experience .2 mg. of TSH was the minimum dose required to elevate the T/S of hypophysectomized rats, Ingbar and Roberts (1952) reported that .0075 mg. of TSH was the lowest effective dose. This discrepancy can probably be explained if one takes into account that these authors, as their abstract appears to indicate, treated their rats chronically with PTU prior to the injection of TSH. The experiment in which TSH was administered to hypophysectomized rats, with and without accompanying injections of PTU, is of particular interest inasmuch as it shows a spectacular potentiation by PTU of the T/S response to a dose of TSH which, both alone and together with PTU, failed to restore completely the morphological picture of the thyroid (Fig. 5). On the basis of these findings the similar dichotomy between inactive thyroid histology and "goitrous" T/S values in the cited studies of Greer (1951, 1952) lends itself to a more conservative explanation than Greer's hypothesis of two thyrotrophins. We merely have to assume that the hypothalamic lesions or the transplantation of the anterior lobe in Greer's studies deprived the adenohypophysis of its ability to react to PTU with a secretion of sufficient amounts of TSH to cause morphological changes in the thyroid. Since, however, small amounts of TSH still may have been secreted it is easy to conceive that PTU potentiated the response of the T/S to this endogenous TSH and thus led to the strikingly high T/S values.

The question arises whether, as Stanley and Astwood (1949) proposed, the effect of TSH on thyroidal iodide trapping is one of the primary functions of the hormone, which is selectively potentiated by PTU, or whether the changes which TSH brings about in thyroid iodide collection are secondary to some other alteration in thyroid iodine metabolism. Rawson (1949), observing that the increase in radioiodine collection by the chick thyroid after the administration of TSH lagged far behind the TSH-induced purge of iodine from the gland, suggested tentatively that the primary effect of this hormone might be on iodine release and other responses of the thyroid to TSH might actually be secondary to the iodine depletion. If our problem is viewed from this angle, it should be remembered that goitrogen treatment, which raises the T/S (Vanderlaan and Vanderlaan, 1947), depletes the thyroid of iodine (Astwood and Bissell, 1944), whereas hypophysectomy, which reduces the T/S, increases the concentration of iodine in the thyroid (Chaikoff and Taurog, 1949). Furthermore, it is of interest to recall that Vanderlaan and Greer (1950) found no change in the thyroid I^{127} concentration when either PTU or TSH alone was administered to hypophysectomized rats, but a marked depletion of iodine when the two substances were given conjointly. Accordingly, in our experiments the greatest rise in the T/S occurred after combined treatment with PTU and TSH. However, although Astwood and Bissell (1944) have demonstrated that the low thyroid iodine content of thiouracil treated rats does not increase substantially after hypophysectomy, Vanderlaan and Greer (1950) have reported a rapid drop in the T/S when PTU-fed rats were hypophysectomized. It can thus be concluded that thyroidal iodide trapping can not be regulated exclusively by the iodine concentration in the gland. Another possibility is that fluctuations in the T/S are secondary and proportional to changes in the rate of iodine discharge from the thyroid, since a dependence of this function on TSH has been clearly demonstrated (Wolff, 1951, Albert and Tenney, 1951). The temporal sequence of events is not incompatible with such a hypothesis since the latency of the T/S response to TSH in hypophysectomized rats appears to be definitely longer (5–6 hours, Fig. 3) than the 2–3 hour latent period which Wolff (1951) found for the reaction of the iodine discharge mechanism. On the other hand, it has been observed that thyroid iodine loss occurs at a lower rate after hypophysectomy (Randall *et al.*, 1951) than after thyroxin treatment (Albert and Tenney, 1951), whereas in our experience the T/S is depressed more by thyroxin treatment than by hypophysectomy. However, it has to be taken into consideration that Albert and Tenney only gave 10 μg. of thyroxin daily while we obtained our marked T/S depression by 200 μg. per day. The rate of thyroid I^{131} loss measured by Wolff and by Albert and his associates was interpreted as an index of the thyroxin output. However, an accordingly rephrased hypothesis suggesting that T/S changes are secondary to alterations in the rate of thyroxin release from the thyroid is clearly untenable if one considers that the conspicuous drop of the T/S which occurs after hypophysectomy in chronically PTU-treated rats (Vanderlaan and Greer, 1950) cannot be caused by a similarly striking reduction in the rate of thyroid hormone discharge. Prolonged treatment with goitrogens largely depletes the thyroid of iodine (Astwood and Bissell, 1944) and consequently leads to an eventual severe slowdown of thyroxin secretion which can hardly be further exaggerated by hypophysectomy.

In conclusion, thyroidal iodide trapping may be a function influenced by some change(s) in thyroid metabolism other than the ones discussed, or it may be directly regulated by TSH, with PTU exerting a selective potentiating effect on the response. TSH is not the only factor which affects the concentration of iodide by the thyroid, since thyroxin was found to lower the T/S even in the absence of the pituitary (Fig. 1), and the blocking influences of thiocyanate (Vanderlaan and Vanderlaan, 1947) and other monovalent anions (Wyngaarden *et al.*, 1952) on the thyroid iodide trap are well known.

SUMMARY

1. The thyroid:serum iodide concentration ratio (T/S) of normal rats was found to be approximately 25:1. Hypophysectomy lowered this ratio to roughly one-fourth (6:1), and prolonged treatment with thyroxin to values approaching 1:1. The T/S of hypophysectomized rats could be further diminished by the administration of thyroxin. Exogenous TSH elevated the T/S of hypophysectomized rats.

2. The response of the T/S of hypophysectomized rats to the injection of a single dose of TSH had a latency of around 5–6 hours, reached a peak between 24–48 hours and had largely subsided by 72 hours.

3. When the 34 hour response of the T/S of hypophysectomized rats to single doses of TSH was examined, a sigmoid log dose-response curve was obtained, with the rectilinear portion lying in the range of .2 to 20 mg. TSH.

4. Both the sensitivity and the slope of the response were enhanced when propylthiouracil (PTU) was injected together with the TSH. Thyroxin, on the other hand, had no demonstrable effect on the T/S rise elicited either by a single dose or by more prolonged administration of TSH.

5. Extracts of transplanted mouse pituitary tumors (Furth) were found to have a high TSH content as judged by their ability to raise the T/S of hypophysectomized rats.

6. No summation of the T/S response was found, when TSH was injected repeatedly.

7. Prolonged administration of TSH to hypophysectomized rats in amounts which were not sufficient to restore the normal morphological appearance of the thyroids elevated the T/S values somewhat above normal. When the same dose of TSH was given together with PTU, there was little change in the histological response of the thyroid, but the T/S-raising effect of the hormone was greatly potentiated. PTU alone had a questionable influence on the T/S of hypophysectomized rats.

8. The mechanism whereby TSH influences thyroidal iodide collection is discussed.

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THE INACTIVATION OF POSTERIOR PITUITARY ANTIDIURETIC HORMONE BY LIVER EXTRACTS[1]

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INACTIVATION of the posterior pituitary antidiuretic hormone at some site in the body has been postulated by Ingram, Ladd and Benbow (1939) on the basis of the small amount excreted after injection of posterior pituitary preparations. The *in vitro* inactivation studies of Heller and Urban (1935) and the *in vivo* observations of Eversole, Birnie and Gaunt (1949) support the existence of such a system and indicate that the greatest activity is found in liver tissue.

The inactivation of other posterior pituitary fractions, namely, the oxytocic (Gulland and Macrae, 1933) and pressor (Møller-Christensen, 1951) by the tissues have been reported. These reports, however, have not described the nature of the inactivating system.

The present investigation was undertaken for the purpose of securing additional information regarding the nature of the system in rat liver homogenates which inactivates posterior pituitary antidiuretic hormone. Some of these observations have been reported in an earlier abstract (Birnie, 1950) and indicate the enzymatic nature of the inactivating system.

METHODS

Homogenates of tissues were prepared immediately after their removal from animals sacrificed by a blow on the head. Prior to removal of the tissues the dorsal aorta and hepatic vein were cut and as much blood as possible was drained from the carcass. The extracts were prepared from 1.0 gram weighed samples of liver in a "Parallel Grind Homogenizer" with 10 volumes of cold distilled water. The homogenates were centrifuged and then filtered through fritted glass to remove all intact cellular material. Homogenates were adjusted to pH 6.5 by means of phosphate buffers. The destruction of posterior pituitary preparations was estimated by the effect of incubation mixtures containing known amounts of vasopressin (Pitressin) on the rate of diuresis of rats given one dose of water. (3.0 ml. per 100 sq. cm. of body surface). In the standard procedure the incubation mixture consisted of 1.0 ml. of tissue homogenate plus 100 milliunits (1.0 ml.) of vasopressin and 3.0 ml. of phosphate buffer. After incubation for 30 minutes at 37° C. 1.0 ml. of this mixture was injected intraperitoneally into test rats. The rats

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used for this diuresis test were fasted but allowed access to water for 18 hours prior to receiving a water load by stomach tube. The rate of urine excretion was followed at 30 minute intervals for a period of 180 minutes.

Urine sodium and potassium determinations were made by means of an internal standard flame photometer.

Blood pressures were determined according to the technique described by Kersten *et al.* (1947). The instrument used was a photoelectric tensometer model FAD-1 manufactured by Metro Industries.

RESULTS

A wide variety of tissues was found to possess systems capable of inactivating vasopressin during *in vitro* incubation. In the rat the greatest activity was found in homogenates of liver tissue (Table 1).

TABLE 1. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN (PITRESSIN) INCUBATED WITH HOMOGENATES OF TISSUES FROM RATS

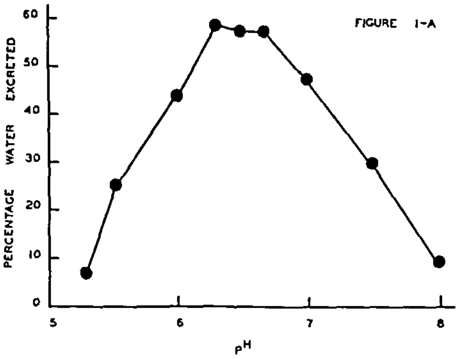
Tissue	Number animals	Percentage of administered water excreted at 90 minutes
		±S.E.
Fresh liver	39	54 ±3.53
Heated liver	26	8 ±2.83
Spleen	12	21 ±3.65
Kidney	12	22 ±4.20
Whole blood	11	12 ±3.50
Skeletal muscle	12	16 ±2.21
Untreated controls	26	52 ±1.80

The vasopressin-inactivating system appears to have the properties of an enzyme. It is thermolabile, exposure to 80° C. for 10 minutes resulting in a complete loss of activity. The active principle is nondialyzable. When liver homogenates were dialyzed against ice-cold 1.0 per cent sodium chloride solution for 2 hours there was little or no loss of vasopressin inactivating ability. Dialysis for longer periods (4 or more hours) resulted in a loss of activity (Table 2). In all reactions tested the precipitation of proteins from the extracts was associated with a loss of vasopressin inactivating

TABLE 2. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN BY RAT LIVER HOMOGENATES WHICH HAD BEEN SUBJECTED TO VARIOUS EXPERIMENTAL PROCEDURES

	Number animals	Percentage of administered water excreted at 90 minutes
Untreated	10	51 ±3.60
Dialyzed 2 hours	7	47 ±4.20
Dialyzed 6 hours	8	33 ±3.67
Half saturation with ammonium sulphate	8	11 ±2.10
Addition of zinc sulphate (0.0005 M)	8	10 ±4.30
Addition of copper sulphate (0.0005 M)	8	8 ±3.52

ability. This loss of activity may be due to the fact that extracts left standing for longer than four hours show some decrease in activity. When the reaction of the homogenates was adjusted to pH 5.2, activity was almost completely destroyed. Further, half saturation with ammonium sulphate results in complete loss of activity (Table 2). The addition of zinc sulphate or copper sulphate (final concentration = 0.0005 M) to the incubation mixture resulted in a complete loss of inactivating ability. During incubation maximum activity was observed between pH 6.2 and 7.0 (Fig. 1-A). The rate of inactivating of vasopressin increases sharply with a rise in temperature from 0° to 37° C.; however, with further increases in temperature there is a marked decline in activity (Fig. 1-B). Under optimal conditions 1.0 ml. of a 10 to 1 aqueous extract of rat liver tissue is capable of inactivating 100 milliunits of vasopressin during a 30 minute incubation period at 37° C.



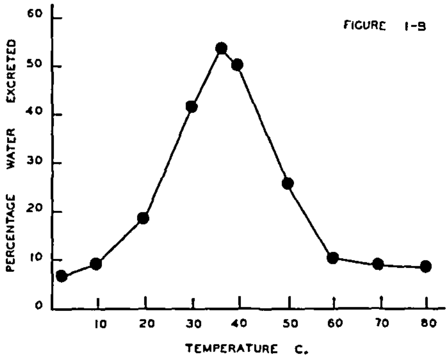


Fig. 1-A. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated at different pH levels. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

Fig. 1-B. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated for 30 minutes at different temperatures. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

No attempt has been made to determine the specificity of the vasopressin inactivating system nor to exclude the possibility that a nonspecific peptidase is involved.

It was observed that homogenates of rat liver tissues destroyed the pressor activity of vasopressin. Incubation mixtures containing homogenates of normal liver tissue failed to cause a rise in blood pressure of test rats while those made with heated liver extracts produced a marked increase in blood pressure (Table 3).

The inactivating system is not confined to tissues of the rat. Fresh human liver tissue contains a potent vasopressin inactivating system. It

TABLE 3. INACTIVATION OF THE PRESSOR ACTION OF VASOPRESSIN BY INCUBATION WITH RAT LIVER HOMOGENATES

	Number animals	Mean control blood pressure, mg. Hg	Mean blood pressure following injection, mm. Hg	Mean change in blood pressure, mm. Hg
Heated liver plus 20 mμ Pitressin	12	122	153	31
Fresh liver plus 20 mμ Pitressin	21	116	117	1
Saline plus 20 mμ Pitressin	15	122	142	20

was observed that the extracts of human liver (sample of tissue removed during abdominal surgery) not only destroyed the antidiuretic activity but also the natriuretic activity of vasopressin (Table 4).

TABLE 4. INACTIVATION OF ANTIDIURETIC AND NATRIURETIC ACTION OF PITRESSIN BY HUMAN LIVER

	Percentage water excreted at 90 minutes	Total sodium excreted at 90 minutes (meq. $\times 10^{-4}$)
Untreated controls	56.1	628
Fresh human liver (20 mμ Pitressin/0.02 g. tissue)	61.4	731
Heated human liver (20 mμ Pitressin/0.02 g. tissue)	8.4	1255

NOTE: All animals received one dose of water by stomach tube (4 ml. per 100 sq. cm. of body surface).

When extracts were made of liver taken from rats which had been given daily injections of 0.05 ml. of carbon tetrachloride in 0.05 ml. of paraffin oil for a period of 12 days, there was a sharp decline in the ability to inactivate vasopressin as compared with untreated controls (Table 5).

Extracts made of liver taken from rats which had been adrenalectomized for from 5 to 7 days showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin as compared with sham operated

TABLE 5. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN INCUBATED WITH RAT LIVER HOMOGENATES

Treatment of donor rats	Number animals	Percentage of administered water excreted at 90 minutes
		±S.E.
Untreated	26	54 ±2.22
CCl_4 injected 12 days	12	39 ±3.69
Adrenalectomized 5 days	21	41 ±2.80
Adrenalectomized 9 days	15	31 ±2.40
Dehydrated 24 hours	10	56 ±3.51

controls. Liver obtained from animals in the terminal stages of adrenal insufficiency showed even less activity (Table 5).

DISCUSSION

The experiments reported here show that cell-free extracts of liver tissue contain an enzyme like system capable of inactivating the antidiuretic, pressor and natriuretic principles of posterior pituitary preparations (vasopressin). The deduction of its enzymatic nature is based upon thermolability, nondialyzability, protein nature, and the kinetics of its reactions. The specificity of the system has not yet been determined. It is possible that the enzyme is a peptidase but thus far it has not been identified.

The distribution of the inactivating system in various tissues confirms the observations of Heller and Urban (1935); Jones and Sclapp (1936); and Eversole, Birnie and Gaunt (1949) on the role of the liver in destroying the antidiuretic principle of posterior pituitary extracts.

The inactivating system is not confined to the tissues of the rat as the present study demonstrates its existence in human liver and previous investigations have found it to be present in the mouse (Birnie, Blackmore and Heller, 1952) and rabbit (Møller-Christensen, 1951).

Whether the system plays a physiological role in the destruction of endogenous posterior pituitary hormone has still not been definitely established. The observation that physiological alterations cause a change in the rate of inactivation suggests that the system may play a role in controlling water metabolism in the intact animal.

It has long been postulated that water retention observed following clinical and experimental liver damage is due, at least in part, to a decreased rate of inactivation of the antidiuretic hormone by damaged liver tissue (Ralli *et al.*, 1945; Labby and Hoagland, 1947). The observations reported here are consistent with such a hypothesis. That these observations do not explain entirely the water retention associated with liver damage is suggested by the observations of Shorr and Zweifach (1948) that the damaged liver itself may produce antidiuretic materials.

It is well established that adrenalectomized animals show a deficient diuretic response to administered water; this has been ascribed in part to an accumulation of antidiuretic material in the body fluids (Birnie *et al.*, 1949; Gaunt *et al.*, 1950). It has been postulated that his increase may be due to (a) the neurohypophysis being hyperactive following adrenalectomy, or (b) a decrease in the normal destruction of antidiuretic material by the tissues. The former possibility is open to serious question on theoretical grounds (Gaunt, 1951) and the present study provides evidence of a decreased destruction of antidiuretic material following adrenalectomy. While failure of the liver to inactivate antidiuretic material would not explain all of the alterations in water metabolism associated with adrenal insufficiency, it is possible a contributing factor to the syndrome.

SUMMARY

1. Cell free extracts of rat liver tissue contain an enzyme-like system capable of inactivating the antidiuretic, pressor, and natriuretic principles of posterior pituitary preparations (vasopressin).

2. Extracts of liver tissue inactivated vasopressin more effectively than did extracts of spleen, kidney, skeletal muscle or whole blood.

3. Extracts made of liver taken from adrenalectomized rats showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin.

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THE ACCELERATING EFFECT OF ADRENALECTOMY ON REGROWTH OF HAIR IN THE THYROIDECTOMIZED RAT

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THE accelerating effect of adrenalectomy on hair growth in the rat is a well established fact, data on which have been reviewed by Baker (1951). This is a curious phenomenon for which there is no ready explanation. If hair growth is a part of general somatic growth, it would be expected to be altered with changes in the pituitary acidophils that secrete growth hormone (somatotrophic hormone, STH). A method for depleting the pituitary of acidophil granules is by thyroidectomy, after which there is known to be retardation of the hair growth. One might expect the loss of acidophil granules in the thyroidectomized rat to prevent the accelerated growth of hair after adrenalectomy.

This hypothesis was tested by the experiments to be reported here; the results, however, were found to be the direct opposite of those anticipated. This unexpected finding was clear-cut and constant.

METHODS

For the experiments were used groups of rats of a colony of Wistar albino strain, of the same sex, usually litter-mates, sometimes non-litter mates of approximately the same age, as follows:—(1) thyroidectomized rats with their adrenals intact, (2) adrenalectomized rats with their thyroids intact, (3) rats in which both thyroidectomy and adrenalectomy had been performed, (4) normal controls.

The rats that were thyroidectomized were operated on at 29 to 109 days of age. During the course of the experiments, evidence that thyroidectomy had been adequately performed was furnished by the retarded body-weight curve and the retarded regrowth of hair after shaving. On termination of the experiment, the characteristic changes in the pituitary (basophilic thyroidectomy cells containing intracellular hyaline material, and absence or marked diminution in granulated acidophils) was the final proof of thyroidectomy.

Bilateral adrenalectomy was carried out through the usual lumbar incision 34 to 222 days after thyroidectomy, thus allowing for the slow decay period of thyroid hormone in the circulation. From the experience of previous experiments (Zeckwer 1935), a long time interval was allowed for the slow development of cellular changes in the anterior pituitary following thyroidectomy. Adrenalectomized rats were given 1% NaCl

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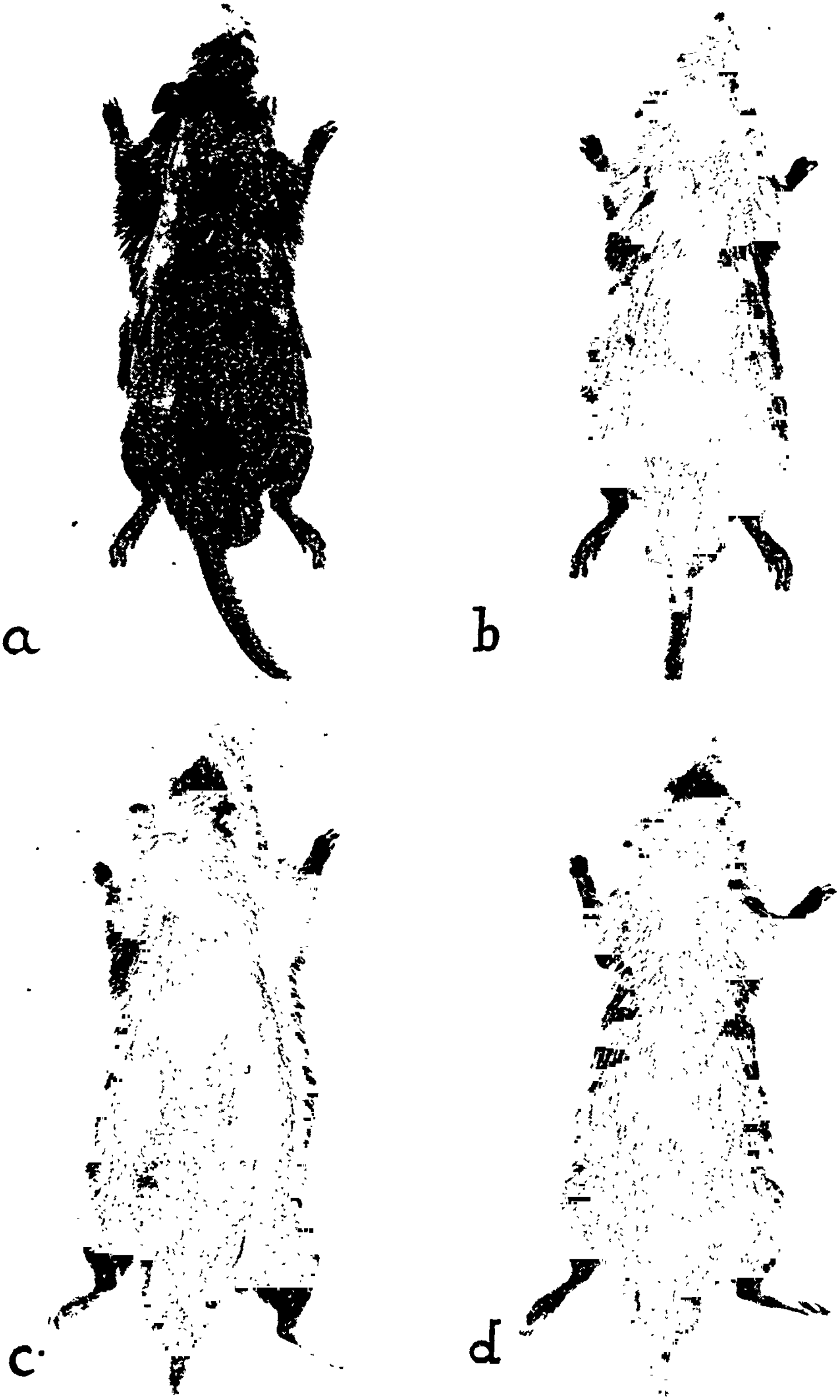
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drinking fluid. During the course of the experiments completeness of adrenalectomy in the rats with thyroids intact was attested by the much more rapid regrowth of hair after shaving, and at the termination of the experiment, proof of complete removal of adrenal tissue consisted of development of adrenal insufficiency on withdrawal of salt solution. At suitable time intervals the hair was shaved by electric clippers from the back and sides of all rats of a group, and the regrowth recorded by drawings and photographs. The same rats were shaved repeatedly, so that data could be obtained at varying time intervals before and after adrenalectomy. Periods up to 47 days after adrenalectomy were studied.

The rats were fed Purina dog chow and were kept in cages with solid sides and floors, open only at the top. Consequently the shaved rats were not exposed to draughts.

For histological studies, sections were prepared from the skin after fixation in formalin and stained with hematoxylin and eosin. The pituitaries were fixed in Helly's solution and stained with Mallory's acid fuchsin-aniline blue, orange G method.

It was necessary to exclude from the analysis of the results: (a) a few very dwarfed rats thyroidectomized for a long period that survived adrenalectomy only a short time, (b) some thyroidectomized rats that had abnormalities of tooth formation which prevented adequate mastication and caused malnutrition, (c) a few adrenalectomized rats that did not develop adrenal insufficiency on withdrawal of salt. When a rat had to be excluded the remaining rats were compared with other groups of rats. In addition, sometimes rats served as their own controls when growth of hair in rats (with and without thyroid glands) was recorded before and after adrenalectomy. There remained for comparison 20 thyroidectomized rats with adrenals intact, 26 adrenalectomized rats with thyroids intact, 25 rats with combined thyroidectomy and adrenalectomy and 23 normal controls.

RESULTS

In normal control rats, regrowth of hair after shaving occurred in regular patterns and waves which have been recorded by various investigators, and details of which have been reviewed by Butcher (1934, 1951), Haddow (1945), and Wolbach (1951). This regrowth occurs symmetrically along a longitudinal axis and leaves certain areas of skin bare until the wave of growth approaches them. The location of the bare areas depends upon the time in the cycle when shaving was done. These cycles succeed each other rapidly in young rats; in older rats they progress slowly.

After adrenalectomy in rats with thyroids intact, this regular pattern of regrowth is usually not perceptible. Instead, there is a rapid diffuse

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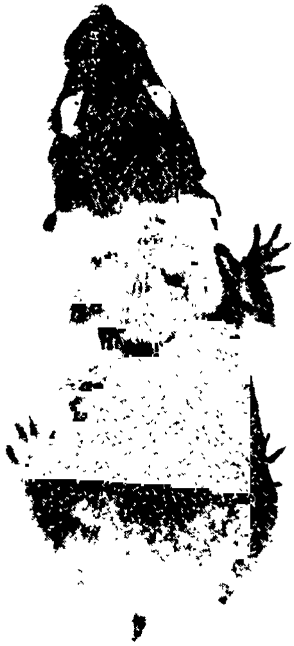
FIG. 1. Regrowth of hair, 30 days after shaving, in male litter-mate rats (49B). (a) Rat at 142 days after thyroidectomy (operation at 43 days of age). Body weight 228 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 142 days after thyroidectomy (operation at 43 days of age) and 35 days after bilateral adrenalectomy. Body weight 233 grams. In contrast to the appearance shown in the preceding figure (a) thick diffuse regrowth of hair has taken place. (c) Intact rat, showing normal patterns of hair growth, alternating with symmetrical bare areas. Body weight 288 grams. (d) Rat at 35 days after adrenalectomy. The operation was done at the same time as in rat (b). Body weight 284 grams. Thick diffuse growth of hair has occurred.



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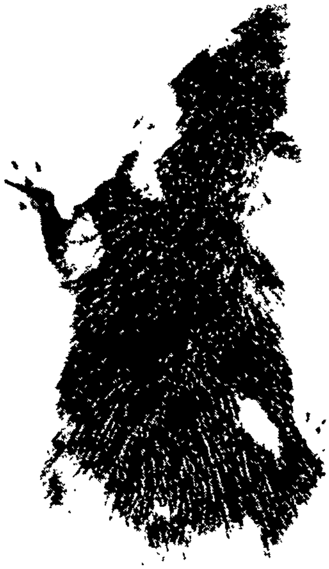

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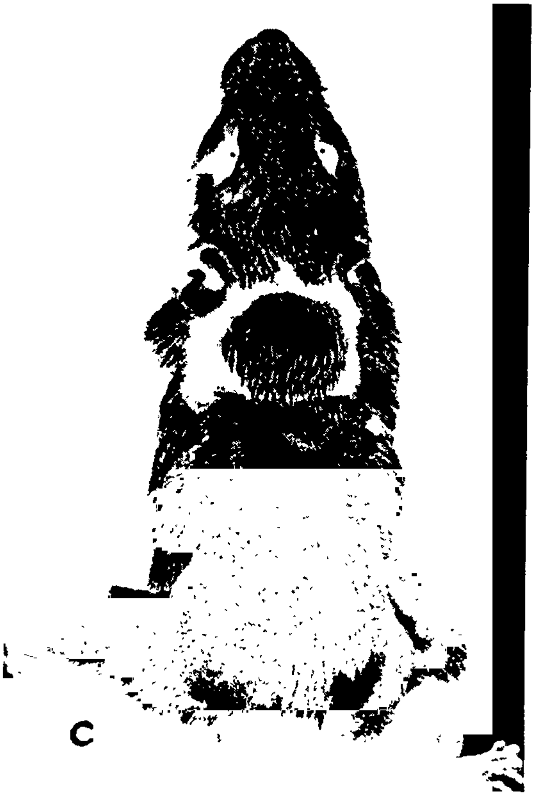

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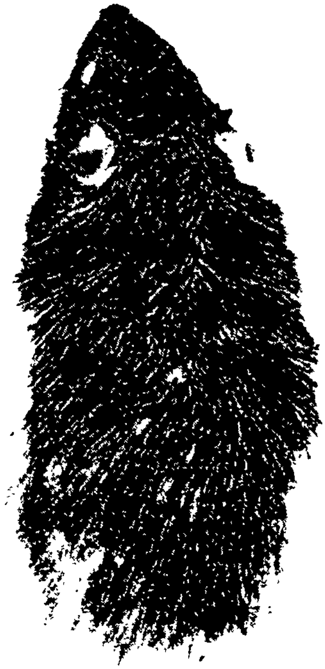

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growth that occurs almost simultaneously over the entire shaved area. This effect did not appear immediately after adrenalectomy but required from 12 to 23 days for development. Once this latent period was passed, diffuse regrowth occurred rapidly after each shaving in a few days' time.

In thyroidectomized rats with adrenals intact, after about 5 weeks following operation (when thyroidectomy changes in the cells of the pituitary are extensive), the regular waves of regrowth occurred in normal patterns, but at a greatly reduced rate, and in many severely dwarfed rats that were thyroidectomized at an early age, the shaved areas remained entirely bald for long periods.

The effect of adrenalectomy in such previously thyroidectomized rats was striking and invariably the same. Adrenalectomy caused a diffuse regrowth of hair, in marked contrast to the condition of the same rat before adrenalectomy and in marked contrast to the appearance of control thyroidectomized rats with adrenals intact. However, this diffuse regrowth was much slower in development than in the adrenalectomized rats with thyroids intact. The latent period was at least one week longer. Eventually, the fur was thick and heavy and long in all adrenalectomized rats whether the thyroid was absent or intact, even in the case of extremely dwarfed long-time thyroidectomized rats that were losing weight. Figures 1 and 2 illustrate the external appearance of 2 experiments on litter-mates.

Microscopic sections of the skin of the rats show changes corresponding to the gross appearance of the hair. In the thyroidectomized rat the hair follicles are small and inactive and occur only near the surface. The dermis is very thin, and adipose tissue occurs between the muscle and dermis. In the thyroidectomized rat after adrenalectomy the follicles have become large and active and have grown deeply, down to the muscle. The dermis has thickened and adipose tissue has disappeared. The section of skin of a normal control rat, taken through a patch of hair growth, shows large active follicles. The adrenalectomized rat with thyroid intact shows good growth of many follicles.

Growth curves of the group of rats that were illustrated in Figure 2, are shown in Figure 3. It is evident that the thyroidectomized rats showed retarded growth.

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FIG. 2. Regrowth of hair, 26 days after shaving, in male litter-mate rats (49R). (a) Rat 154 days after thyroidectomy (operation at 29 days of age). Body weight 172 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 154 days after thyroidectomy (operation at 29 days of age) and 26 days after bilateral adrenalectomy. Body weight 158 grams. Thick diffuse regrowth of hair has taken place. (c) Intact rat, showing symmetrical patterns of regrowth, alternating with bare areas. Body weight 282 grams. (d) Rat at 26 days after bilateral adrenalectomy. This operation was done at the same time as in rat (b). Body weight 290 grams. Diffuse thick growth of hair has occurred.

In microscopic sections the pituitary of the thyroidectomized rat and the rat with combined thyroidectomy and adrenalectomy have identical changes; namely, granulated acidophil cells have disappeared entirely in both. Each pituitary is filled with large basophilic thyroidectomy cells, many of which contain intracellular hyaline material. These thyroidectomy cells are regarded as cells producing TSH which is secreted in excess when the pituitary is released from the inhibiting effect of thyroxin.

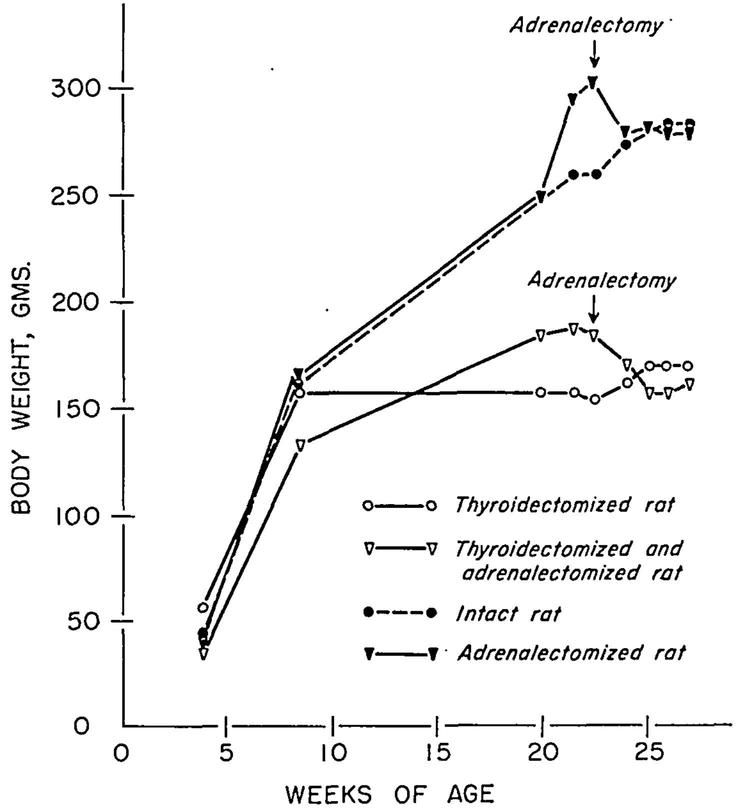

FIG. 3. Growth curve of litter-mate rats (49R) that are illustrated in Figures 2 and 3.

DISCUSSION

It is of interest to attempt a correlation of the structural with the functional changes in the pituitary. Several questions arise: Is the disappearance of granulated acidophils in the pituitary of the thyroidectomized animals a manifestation of decreased secretion of STH ? Does this mean that STH secretion is influenced by thyroid activity? Are the numerous acidophils in the pituitary of the adrenalectomized rats responsible for accelerated hair growth? Unfortunately, a simple explanation that body growth and hair growth are both manifestations of STH and are both correlated with numbers of acidophils does not hold because we find that accelerated hair growth occurs in rats with combined thyroidectomy and adrenalectomy in which no granulated acidophils are present in the pituitary.

In spite of this apparent inconsistency, there is evidence accumulating from biochemical studies that growth hormone secretion is altered after thyroidectomy and after adrenalectomy.

That there may be an increased amount of growth hormone in adrenalectomized animals is indicated by the experiments of Engel (1951b) who states that in the adrenalectomized animal there is an increased rate of protein synthesis and a decreased rate of protein breakdown and thus presumably the adrenalectomized animal should show a greater growth rate than normal, if it were not obscured by reduced appetite of the animal. Analyses of the carcasses of normal rats restricted to the same food intake as adrenalectomized animals suggested chemical growth in the latter. He cites a thesis by J. Winternitz (1942) indicating that specific tissues may show enhanced growth rates after adrenalectomy.

Tepperman, Engel, and Long (1943) also cite Winternitz' experiments (1942) as suggesting that the removal of the adrenals which normally promote protein catabolism results in a relative hyperactivity of the anterior pituitary in promoting anabolism.

Wyman and Tum Sudan (1943) found increased growth of the epiphyseal cartilage of adrenalectomized rats, interpreted to mean that adrenalectomy releases growth hormone from the pituitary. It is of interest that they found that this increased growth of cartilage began near the end of the 2nd week after adrenalectomy, i.e. exactly at the time that elapsed before hair growth was accelerated in the present experiments.

Other biochemical studies seem to have a bearing on the question of whether growth hormone production is altered after thyroidectomy and after adrenalectomy. Hoberman (1950) in studies of amino acid and protein metabolism gives data which suggest the possibility that the secretion or synthesis of growth hormone is influenced by the activity of the thyroid gland, and he suggests that "in hypothyroidism the rapidity of breakdown of amino acids is due to a release of the catabolizing system from the inhibiting influence of growth hormone." He states that "adrenalectomy appears to be responsible for stimulation of protein synthesizing reactions whether performed on normal or hypothyroid rats" and he finds that "the nitrogen output is significantly lower in the absence of both adrenal and thyroid glands than after extirpation of either gland alone."

Is hair growth an expression of growth hormone (STH) activity? Hypophysectomy causes great retardation in regrowth of hair (Snow & Whitehead, 1935). We should not expect hair growth to be completely abolished by hypophysectomy, because, as Engel (1951a) suggests, it is generally recognized that "hormones do not initiate or abolish any specific metabolic reaction, but simply modify the rate of existing ones." Both hair growth and body growth are suppressed by administration of ACTH or cortisone to the normal animal, so one would expect the growth of both would be increased after adrenalectomy, if the effects of growth were not masked by decreased food intake.

Van Dyke *et al.* (1950) suggest that "production of growth hormone by the pituitary is not under the control of the blood level of growth hormone." The evidence summarized in the present report suggests the possibility that the level of adrenal cortical hormone in the blood may be a means of controlling the rate of growth hormone secretion. Thus removal of the adrenals would remove an inhibition to the pituitary and release growth hormone secretion.

In the homeostatic regulation of carbohydrate metabolism, it is well established that the hypoglycemic effect of insulin secretion by the pancreas is counterbalanced by the 2 opposing factors of (1) anterior pituitary diabetogenic factor and (2) the adrenal cortical factor promoting gluconeogenesis. If the adrenal cortical factor is removed by adrenalectomy, one might anticipate that the anterior pituitary might compensate by secreting extra diabetogenic factor. Consistent with this idea is the fact that adrenalectomized rats, if given sufficient NaCl, will tend to maintain adequate levels of blood sugar. Since the anterior pituitary diabetogenic factor has been attributed to growth hormone STH, one might postulate that after adrenalectomy there might be compensatory increased secretion of growth hormone.

We should not expect accelerated hair growth to be demonstrable in such animals as dogs and cats in which fatal adrenal insufficiency occurs too rapidly.

Aside from these theoretical considerations, the observation of hair regrowth after shaving offers a very simple method for estimating the adequacy of thyroidectomy or adrenalectomy during the course of long time experiments, and may also give an estimate of rate of STH secretion even when body weight can not be taken as an index, due to inadequate food intake.

The lack of correlation between granulated acidophils and apparent STH secretion can not be explained at present. The absence of acidophil granules does not necessarily mean that no STH secretion is produced. The actively secreting cell discharges granules and then the cell reverts to the chromophobe appearance. Lack of granules may mean that there has been an accelerated rate of secretion by the acidophils to the point of exhaustion, where all hormone is discharged into the circulation and none is stored in the cell. Secretion rate and storage need not run parallel.

Whatever is the cause of the accelerated hair growth after adrenalectomy it is obvious that the changed metabolism takes several weeks to develop. This change, once established, is maintained for long periods. In the present experiment, no point was reached at which the acceleration of growth of hair disappeared.

Ralli and Graef (1945) reported that DOCA prevented the stimulating effect of adrenalectomy on regrowth of hair. At present it does not seem possible to place this finding in relation to the results of the present experiments.

A study of hair growth after combined thyroidectomy and adrenalectomy is recorded by Butcher (1941) who devised experiments to find out if accelerated hair growth after adrenalectomy was accomplished by thyroid secretion. He used very young rats, underfed so as to reduce the rate of hair growth, and thyroidectomized at 36 days of age. In some rats adrenalectomy was done at the same time, in others adrenalectomy was done 7–10 days and 3 weeks after thyroidectomy. He concluded that thyroidectomy had no effects on hair growth after adrenalectomy. However, his experiments do not extend over a sufficiently long period to give time for adequate decay of thyroid hormone in the circulation, nor could advanced pituitary changes take place. Furthermore, the periods of hair growth he studied were only 8 or 9 days. In brief, in Butcher's experiments, conditions were very different from those in the present experiments.

SUMMARY

Regrowth of hair after shaving was studied in groups of rats of the same sex and age: (1) rats thyroidectomized at 29 to 109 days of age; (2) rats thyroidectomized at the same time as in the preceding group and also adrenalectomized 34 to 163 days after thyroidectomy; (3) rats adrenalectomized at the same time as in preceding group; (4) intact controls.

Confirming previous studies, it was found that thyroidectomy retarded regrowth of hair.

In adrenalectomized rats, with their thyroids intact, there was a latent period of 12 to 23 days, after which hair grew diffusely instead of in the normal cycles, and at an accelerated rate. After adrenalectomy in previously thyroidectomized rats, there was a longer latent period of at least one week more than in those with thyroids intact, followed by a diffuse regrowth of hair that eventually equalled the growth of hair in adrenalectomized rats with thyroids intact. Microscopic appearance of skin was consistent with the gross findings.

After thyroidectomy, pituitaries showed the usual disappearance of acidophils and development of basophilic "thyroidectomy" cells. Subsequent adrenalectomy did not reverse these histological changes; that is, acido-

philic cells did not reappear. Nevertheless, from the results of other investigators, it seems likely that adrenalectomy does cause the pituitary to form more growth hormone.

In view of the fact that STH is commonly ascribed to the acidophil cells of the pituitary, the absence of acidophils in rats with combined thyroidectomy and adrenalectomy, in which accelerated regrowth of hair occurred, is an apparent inconsistency for which there is no explanation at present.

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A RAPID METHOD FOR THE QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE PHYSIOLOGICAL ACTIVITY OF THE ADRENAL CORTEX[1]

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IT IS apparent to investigators interested in the physiology, pharmacology, or physiological chemistry of the adrenal cortical hormones that a rapid method for the qualitative and quantitative analysis of the hormonal secretions of the adrenal cortex would be of considerable value. It is the purpose of this communication to describe such a procedure and to present preliminary data resulting from the application of these techniques.

EXPERIMENTAL

For this particular study, dogs were chosen for the experimental animal. The dogs were sacrificed by an intracardiac injection of a lethal dose of nembutal. The adrenal glands were quickly removed and placed in ice. After removing the connective tissue and fat, the tissues were blotted, weighed, and slices prepared with a Stadie slicer. An aliquot was reserved for nitrogen analysis. The remainder of the slices were incubated in a large Warburg flask containing 20 ml. of the animal's own serum along with 10 mg. of adrenal cortical stimulating hormone (Armour lot #128-105R, potency 1.6 times that of LA-1-A standard), 50,000 units of penicillin, and 0.1 gm. of streptomycin. The vessel was gassed with 95% O_2–5% CO_2; the bath temperature was 37.5° C. and the time of incubation was 20 hours.

Following incubation, the adrenal slices and incubating medium were homogenized and subjected to fractionation similar to that described by Tennent, Whitla, and Florey (1951). The aqueous homogenate was treated with 5 volumes of acetone and the precipitated proteins filtered off. After removal of the acetone by vacuum distillation, the residual aqueous phase was extracted 5 times with ethyl acetate and dried over sodium sulfate. The bath temperature for all distillations was 40° C. The ethyl acetate was

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DISCUSSION

It is clear that following the incubation of relatively small amounts of adrenal cortical tissue, significant quantities of cortical steroid hormones can be recovered by the partition and chromatographic techniques. It is quite evident that the incubating procedure is of importance in estimating the actual elaboration of steroid hormones. The largest quantity of the predominating steroids obtained by direct isolation employing similar chromatographic techniques from beef adrenal glands was 400 μg. per pound of Compounds B and A (Zaffaroni and Burton, 1951). In the present experiments, as much as 100 μg. of Compound F could be recovered after incubating 1 g. of adrenal tissue. In the studies of beef adrenal glands cited (Zaffaroni and Burton, 1951), Compound F was present in the gland in quantities considerably less than Compounds B and A. In the present experiments with dog adrenal slices, Compounds F and E were predominant. It is certainly possible that this may be due to the difference in the species of animals employed (cf. Pfiffner, 1942). The discrepancy between the quantity of hormone present in the gland and the actual physiological elaboration receives support from the experiments of Hechter *et al.* (1951) who found that the chief hormones elaborated by perfused beef adrenal glands were Compounds F and B.

It is hoped that these techniques will be useful in evaluating adrenal cortical function during various stressing conditions. Some experiments along these lines are in progress and will be reported later.

SUMMARY

A relatively rapid method for the qualitative and quantitative measurement of the steroid hormone production of adrenal cortical tissue has been described.

The following steroids have been identified and quantitative estimation of their elaboration determined: Compounds F, E, A, S, and B. Compound F is produced in the greatest quantity; Compound E somewhat less, and smaller amounts of A, S, and B were found.

It is apparent that the steroid hormones recovered after incubation differ greatly in a quantitative manner from those recovered by direct isolation from the adrenal cortex.

ACKNOWLEDGEMENTS

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A QUANTITATIVE METHOD OF DETERMINATION OF THE DIABETOGENIC ACTIVITY OF GROWTH HORMONE PREPARATIONS*

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A NEW, rapid and quantitative method of determination of the diabetogenic activity of growth hormone preparations has been developed, based on the effect of this hormone on obese-hyperglycemic mice. The metabolic characteristics of this new Mendelian recessive condition have been described in previous publications (Bleisch, Mayer and Dickie, 1952; Guggenheim and Mayer, 1952; Mayer, Russell, Bates and Dickie, 1952; Mayer, Russell, Bates, and Dickie, 1952).

Particularly relevant to the question discussed here are the characteristics of the hyperglycemia in the obese mice. This develops as a progressive condition, usually after the 12th and before the 18th week of life. The precise time of onset seems to be conditioned by the presence or absence of genes other than "obese" in the genetic composition of the obese mice. The hyperglycemia is sensitive to the amount and the nature of the diet. It is highly sensitive to growth hormone, being either precipitated (during the prediabetic diet) or very much exaggerated (during the diabetic or hyperglycemic phase) by a single injection of growth hormone. By contrast, the blood sugar of non-obese mice are unaffected even by prolonged, massive treatment. Decreased glucose phosphorylation rates have been implicated in the etiology of the hyperphagia, in the light of the glucostatic theory of regulation of food intake (Mayer, 1952; Mayer and Bates, 1952).

Recent results (Mayer, unpublished) indicate that the obese animals are characterized by hyperfunction of the alpha cells of the pancreas and overproduction of a pancreatic hormone other than insulin. Growth hormone appears to be the trophic hormone corresponding to this new pancreatic factor.

METHOD AND RESULTS

The property of single injections or of a few injections of growth hormone to increase drastically the blood glucose of obese-hyperglycemic animals is the basis of the proposed

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method. Although reliable results were obtained with single injections in diabetic animals, maximum precision and homogeneity was obtained when using obese mice still in the prediabetic period injected for 3 consecutive days with 3 equal doses and effecting the determination on the 4th day. The Folin Ferricyanide colorimetric method (Folin, 1930, 1932) following the Somogyi method of precipitation (Somogyi, 1945) was used. The animals were 3 to 4 months old, weighed 40 to 50 g. and were fed Purina Laboratory Chow. Sex introduces no variable in the determination.

TABLE 1. EFFECT OF THREE CONSECUTIVE DAILY INJECTIONS OF GROWTH HORMONE ON THE BLOOD SUGAR OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Dose (mg. growth hormone)	none (saline)	0.1	0.2	0.5	1	2	4	10
Number of animals	10	8	8	7	5	4	4	4
Mean blood sugar (mg. per cent)	160	207	218	230	248	303	355	507
Standard deviation (mg. per cent)	±20	±12	±9	±6	±3	±4	±15	±32

A standard curve (given in tabular form in Table 1) was established using a usual (Armour) preparation. Doses given are the daily dosage. The results were shown to be independent of the method of preparation by comparing the standard Armour hormone with a preparation made according to the directions of Raben and Westermeyer (1951, 1952) for the isolation of "non-diabetogenic" growth hormone. (Comparison was made on the basis of equal nitrogen retaining activity or growth promotion in the hypophysectomized rat.) Possible errors in the method due to contamination by other pituitary hormones were studied by determining the effect of adding daily large supplements of ACTH and thyrotrophic hormone to the standard growth hormone preparation—ACTH (Armour) even in large amounts did not modify the results. Thyrotrophic hormone (Armour) increased blood sugar values only if doses of the same order of magnitude than growth hormone were used. The preparation used (Armour) did, however, show some growth activity. (Thyroxine, in doses up to 100 μg. per animal does not increase blood sugar levels (Mayer *et al.*, in press).) These results are summarized in Table 2.

TABLE 2. EFFECT OF MODE OF PREPARATION AND OF ADDITION OF OTHER PITUITARY HORMONES ON THE BLOOD GLUCOSE RESPONSE OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Treatment	Number of animals	Mean blood glucose and standard deviation (mg. per cent)
None (saline)	10	160 ±20
0.2 mg. standard Armour growth hormone	8	230 ± 6
0.2 mg. Raben et al. growth hormone	6	225 ±27
0.2 mg. growth hormone+0.2 mg. ACTH	8	220 ± 9.2
0.2 mg. growth hormone+0.2 mg. thyrotropic hormone	6	235 ±15

DISCUSSION

This method is simple, quantitative and speedy. It has been repeatedly used in this laboratory in studies of purification of growth hormone. It may be emphasized again that, while the experimental conditions described represent ideal circumstances for the carrying out of the determination, it is possible, whenever available material is limited, to carry out the determination using single doses. A standard curve should be established with single doses of the reference preparation. Sensitivity, though not reproducibility, is increased when more diabetic mice (older animals) are used and when a high glucose diet is fed to the animals.

Preliminary results, indicating many similarities between goldthioglucose obesity (Waxler and Brecher, 1950) and the hereditary obese hyperglycemic syndrome suggest that goldthioglucose-treated animals, more readily available, may be used in this bioassay.

SUMMARY

A new method for the determination of the diabetogenic activity of growth hormone preparations, making use of the trophic effect of growth hormone on the blood glucose of hereditary obese-hyperglycemic mice is presented. The method is rapid and can be used with hormones prepared by different methods, even in the presence of other pituitary contaminants.

Acknowledgement

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HORMONAL FACTORS INFLUENCING FAT DEPOSITION IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF THE WHITE RAT[1]

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INTRODUCTION

IT HAS already been reported that prolonged exposure to cold leads to a hypertrophy of the interscapular brown adipose tissue in the rat (Pagé and Babineau, 1950). Since in such animals metabolism is necessarily enhanced, it was thought worthwhile to investigate the effects of hyperthyroidism on the brown fat of rats kept at ordinary room temperature. The role of adrenal hormones was also studied in view of the findings of Fawcett and Jones (1949) that the functional integrity of the adrenal cortex is essential for the normal complement of lipids in brown adipose tissue. It has also been reported that during stress, changes in the weight and appearance of this tissue parallel changes in the adrenals (Selye and Timiras, 1949; Lemonde and Timiras, 1951.)

MATERIAL AND METHODS

Young albino male rats of the Wistar strain were used. They were housed in individual cages and fed a purified ration supplemented with crystalline vitamins. This ration contained 18% protein in the form of casein and 4% fat (Mazola corn oil). Thyroid powder (VioBin), when used, was incorporated in the ration at the level of 0.05%. Hormones were administered subcutaneously in the following daily doses: thyroxine-sodium (BDH) 5 mg./kg. of body weight; cortisone acetate (Merck), 2.5 mg.; DCA (Ciba), 2.5 mg; and ACTH (Armour), 2 mg. Thyroxine-sodium was dissolved in dilute sodium hydroxide and 0.9% NaCl was used as a diluent for other hormones when indicated.

Bilateral adrenalectomy was performed in a single sitting through two cutaneous incisions under ether anesthesia. Adrenalectomized rats were given tap water containing 1% sodium chloride *ad libitum*. The animals were sacrificed by decapitation under light ether anesthesia and the interscapular brown adipose tissue was immediately dissected and weighed. It was dried to constant weight *in vacuo* at 48° C. The dry matter was later homogenized in a chloroform-methanol mixture and the fat was extracted therefrom at

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room temperature according to the method of Folch *et al.* (1951). The fresh weight of the perirenal fat (left side) was used as an index of fat depots, Pagé and Babineau (1951) having found the amount of fat in this tissue to be a reliable guide to total body fat.

Statistical treatment was limited to the determination of the standard error by the usual formula where it is equal to $\sqrt{\Sigma d^2/n\ (n-1)}$; $n(n-2)$ was used in groups numbering less than ten animals. The probability of identity between values was calculated from standard tables for Student's t.

RESULTS

Thyroid feeding

Thyroid feeding to young rats for a period of two weeks led to a considerable hypertrophy of the brown adipose tissue. This gain in the fresh weight of the tissue could be entirely accounted for by the increase in fat content since no change occurred in the water or fat-free dry matter by comparison with control rats killed on the same day (Table 1, Experiment 1). It is also seen that fat deposition in the brown adipose tissue was accompanied by a reduction of depot fat as reflected in the size of the perirenal fat. On the other hand, body growth in untreated animals results in a significant increase in all three fractions of the brown fat. This is the general picture which was invariably reproduced in all experiments of this type.

TABLE 1. EFFECT OF THYROID FEEDING ON THE BROWN ADIPOSE TISSUE OF INTACT AND OF ADRENALECTOMIZED RATS

Group	Experiment 1*					Experiment 2†	
	Intact rats			Adrenalectomized rats		Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed
No. rats	10	10	10	11	10	6	7
Mean body wt., gms.							
Initial	145	145	145	145	147	236	226
Final	—	208	183	147	150	302	266
Perirenal fat, mg.	569 ± 50	942 ± 110	410 ± 60	negligible	negligible	1191 ± 300	667 ± 300
P‡		.01	.01				.7
Brown adipose tissue							
Fresh wt., mg.	167 ± 14	284 ± 20	407 ± 21	163 ± 12	197 ± 19	350 ± 32	554 ± 69
P		.01	.01	(.9)§	.25		.02
Water content, mg.	75 ± 5	130 ± 11	130 ± 7	108 ± 9	109 ± 6	172 ± 16	190 ± 9
P		.01	.9	(.01)	.9		.3
Fat content, mg.	60 ± 8	113 ± 15	234 ± 20	24 ± 5	56 ± 15	124 ± 17	313 ± 26
P		.01	.01	(.01)	.05		.01
Fat-free dry matter content, mg.	32 ± 3	41 ± 3	43 ± 5	31 ± 2	32 ± 4	54 ± 7	51 ± 13
P		.05	.8	(.8)	.8		.8

* Experimental period: 2 weeks.
† Experimental period: 36 days; rats thyroid fed during the last 29 days.
‡ P = Probability of identity between the two values shown above.
§ Probability of identity between untreated adrenalectomized rats and controls killed initially.

The effect of adrenalectomy and of thyroid feeding to adrenalectomized rats was studied concurrently. In the case of the non-treated animals, the size of the brown fat at the termination of the experimental period was the same as that of control rats killed initially. Its composition was significantly altered, however, the fat content dropping markedly and being replaced by an equal quantity of water (Table 1, Experiment 1). It might be argued that the stationary weight of the brown adipose tissue was consequent upon the lack of gain in body weight of these animals. However, a similar growth inhibition of this tissue was observed in a subsequent experiment where the adrenalectomized rats registered substantial gains in body weight (Table 2). The results seem to confirm fully the conclusion reached by Fawcett and Jones (1949) and mentioned above.

TABLE 2. EFFECT OF DCA ON THE BROWN ADIPOSE TISSUE OF INTACT AND ADRENALECTOMIZED RATS

Group	Intact rats			Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	DCA*	Controls (end of expt.)	DCA*
No. rats	10	10	10	12	12
Mean body wt., gms.					
Initial	140	141	140	140	140
Final	—	231	226	202	218
Perirenal fat, mg.	277 ±35	1078 ±156	1148 ±159	389 ±95	549 ±60
P		0.01	.7		.2
Brown adipose tissue:					
Fresh wt., mg.	248 ±20	291 ±21	291 ±16	245 ±18	244 ±16
P		.2	.9		.9
Water content, mg.	—	142	126	126	99
Fat content, mg.	—	101	120	74	100
Fat-free dry matter content, mg.	—	48	45	45	45

* Administered during the last two weeks of a three week experimental period.

Thyroid feeding to adrenalectomized rats caused a slight but not significant increase in the fresh weight of the brown fat (Table 1, Experiment 1). This increase was confined to the fat fraction, however, and on this basis, it is significant at the 5% level. Identical results were later obtained in adrenalectomized rats receiving thyroxine subcutaneously (Table 3). In contrast to these findings, when larger rats were adrenalectomized and fed thyroid powder for an extended period of time, there resulted a hypertrophy of the brown fat (Table 1, Experiment 2) comparable in degree and nature to that observed in intact rats similarly treated. It is doubtful if one can exclude in such a prolonged treatment the possible intervention of accessory adrenal tissue or even the cortical-like action of androgens. These same rats were also reasonably well supplied with fat reserves in contrast to the previous ones so that in the smaller rats, limited response to thyroid feeding or to thyroxine administration may have been partly due to lack of mobilizable fat.

Desoxycorticosterone acetate

Results shown in Table 2 indicate that the administration of DCA has no effect on the size of the brown adipose tissue either in intact or in adrenalectomized rats, nor does it affect significantly the amount of depot (perirenal) fat. Analyses of the brown fat were carried out on pooled samples so that the significance of any observed difference cannot be evaluated statistically. It may be noted, however, that in both treated groups there occurs a rise in the fat content of the brown adipose tissue and a concomitant decrease in water, a unique finding in all our studies to date.

Cortisone and thyroxine in adrenalectomized rats

The effect of cortisone in adrenalectomized rats receiving thyroxine or not was next investigated. Adrenalectomized controls received no treatment or were given thyroxine alone (Table 3). It is seen that cortisone alone caused a significant increase in the size and fat content of the brown adipose tissue as compared to the untreated controls. In fact, this hypertrophy is as large as that observed following thyroxine or cortisone treat-

TABLE 3. EFFECT OF THYROXINE, CORTISONE, AND OF THYROXINE+CORTISONE ON THE BROWN ADIPOSE TISSUE OF ADRENALECTOMIZED RATS

Group	Control	Thyroxine	Cortisone	Thyroxine + cortisone
No. rats	10	6	10	10
Mean body wt., gms.				
Initial	115	119	115	116
Final	135	125	132	115
Perirenal fat, mg.	88 ±20	negligible	215 ±30	112 ±20
P vs controls			.01	.4
Brown adipose tissue				
Total fresh weight, mg.	254 ±18	309 ±25	403 ±19	546 ±42
P vs controls		.1	.01	.01
P vs cortisone group		.01		.01
Water content, mg.	152 ±9	162 ±6	169 ±7	174 ±10
P vs controls		.1	.1	.3
Fat content, mg.	62 ±8	99 ±17	182 ±16	316 ±33
P vs controls		.05	.01	.01
P vs cortisone group		.01		.01
Fat-free dry matter content, mg.	40 ±5	48 ±4	52 ± 6	56 ±5
P vs controls		.1	.07	.02

Experimental period: 14 days; thyroxine or cortisone given on the 9th, 10th and 11th day.

ment in intact rats (Tables 4 and 5). When given in conjunction with thyroxine, the increase in weight and fat content is double that obtained with cortisone alone.

These results would indicate that cortisone administration restores the action of thyroxine which is otherwise greatly impeded by adrenalectomy.

Cortisone and thyroxine in intact rats

In intact animals, the action of thyroxine is similar to that obtained by thyroid feeding (Tables 4 and 5). Cortisone has an effect comparable to that of thyroxine with this difference that the water content rises significantly and that the deposition of fat is less pronounced (Table 4). Seifter *et al.* (1951) have also reported an increase in the size and fat content of this tissue in pregnant rats given cortisone. On the other hand, Antopol (1950) working on mice and using relatively larger doses of cortisone observed an atrophy of the brown fat. Sala *et al.* (1951) report that this tissue regresses under the influence of cortisone administered over an extended period of time.

TABLE 4. EFFECT OF THYROXINE AND OF CORTISONE ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Cortisone
No. rats	20	10	8
Mean body wt., gms.			
Initial	135	137	135
Final	170	149	140
Perirenal fat, mg.	436 ±37	288 ±37	328 ±50
P vs controls		.02	.1
Adrenal wt., mg.	23.7 ±0.6	26.6 ±0.6	19.0 ±1.5
P vs controls		.01	.01
Brown adipose tissue:			
Total fresh wt., mg.	326 ±19	461 ±26	426 ±27
P vs controls		.01	.01
P vs thyroxine group			.4
Water content, mg.	156 ±7	157 ±9	177 ±7
P vs controls		.9	.05
Fat content, mg.	124 ±12	254 ±17	190 ±17
P vs controls		.01	.01
P vs thyroxine group			.02
Fat-free dry matter content, mg.	46 ±3	50 ±4	59 ±6
P vs controls		.4	.08

Experimental period: 6 days; thyroxine or cortisone given on the 1st, 2nd and 3rd day.

When cortisone is given in conjunction with thyroxine, an apparently

TABLE 5. EFFECT OF THYROXINE, THYROXINE+CORTISONE AND OF ACTH ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Thyroxine + cortisone	ACTH
No. rats	12	12	12	12
Mean body wt., gms.				
Initial	134	134	134	134
Final	174	153	132	171
Perirenal fat, mg.	411 ±50	90 ±10	100 ±10	421 ±60
P vs controls		.01	.01	.9
Adrenal wt., mg.	23.7 ±0.7	28.1 ±1.2	18.6 ±1.4	25.8 ±0.7
P vs controls		.01	.01	.08
Brown adipose tissue				
Total fresh wt., mg.	310 ±15	441 ±15	544 ±29	367 ±22
P vs controls		.01	.01	.05
P vs thyroxine group			.01	
Water content, mg.	156 ±4	151 ±5	161 ±9	174 ±9
P vs controls		0.5	0.6	0.1
P vs thyroxine group			.3	
Fat content, mg.	107 ±11	240 ±9	333 ±19	134 ±14
P vs controls		.01	.01	.2
P vs thyroxine group			.01	
Fat-free dry matter content, mg.	47 ±1	50 ±3	50 ±6	59 ±4
P vs controls		.3	.6	.01

Experimental period: 8 days; Rats injected daily, the 6th day excepted.

additive response is obtained (Table 5). It is to be noted that cortisone induces an atrophy of the adrenals even in the presence of excess thyroxine. The fact that this hormone is equally potent whether the adrenals are removed or atrophied points to its preponderant role among cortical hormones with respect to fat deposition in the brown adipose tissue.

ACTH in intact rats

The action of ACTH is reported in Table 5. The dosage used was too low to increase significantly the size of the adrenals. Nevertheless, the brown adipose tissue was significantly hypertrophied and a general increase in all three fractions occurred, the rise in fat-free dry matter content being statistically significant. This response differs somewhat from that obtained with thyroxine or cortisone and is more reminiscent of spontaneous changes occurring during body growth (Table 1). Baker, Ingle and Li (1950) have observed a higher fat content of the brown adipose tissue in rats treated for 21 days with ACTH. From the trend shown in Table 5, it can be assumed that a longer treatment would have yielded similar results.

Hormonal treatment and depot fat

With respect to general fat stores, it can be seen that hyperthyroidism led to a considerable decrease in the amount of perirenal fat in all cases. Cortisone, on the other hand, seemed to prevent the loss of fat consecutive to adrenalectomy although it did not affect body weight. It also afforded some measure of protection against fat depletion in thyroid-treated adrenalectomized rats. These results are in accordance with the findings of Stoerk and Porter (1950). In intacts rats, neither cortisone nor ACTH had a perceptible effect on depot fat and the same seems to apply to DCA in intact and in adrenalectomized animals.

DISCUSSION AND CONCLUSIONS

Of chief interest are the findings: (1) that thyroxine elicits a minimum response in the absence of the adrenals; (2) that cortisone is equally effective in intact and in adrenalectomized rats; and (3) that an enhanced response is obtained when the two hormones are combined either in intact or adrenalectomized animals. There thus appears to exist a synergism between cortisone and thyroxine in respect to fat deposition in the brown adipose tissue. It may be that excess thyroxine given alone exhausts the cortisone producing capacity of the adrenal while cortisone given singly inhibits the production of thyroid hormone as some reports indicate (Money *et al.*, 1951; Lederer, 1952).

The origin of this extra fat in the brown adipose tissue remains to be determined. Experiments now underway seem to indicate that it is mobilized from fat stores in other regions. In view of the reported antilipogenic action of cortisone, one would hardly expect this fat to have been synthesized *in situ*. It is difficult to correlate our findings with those of Engel and Scott (1951) regarding the effect of cortisone on glycogen deposition in the brown fat. These authors express the glycogen concentration on a fat basis and should cortisone have stimulated fat deposition, it would follow that glycogen concentration would suffer an apparent decrease unless the whole tissue content in this substance rose proportionately.

Glycogen deposition in the brown adipose tissue is often considered as indicative of fat synthesis (Wertheimer and Shapiro, 1948). However, if the fat laid down in the brown adipose tissue originates elsewhere, one may ponder whether it is later oxidized directly or over the pathway of carbohydrate synthesis. Ingle (1950) has envisaged the possibility of fat conversion to carbohydrate under the influence of corticosteroids and Segaloff and Many (1951) suggest that extra urinary glucose in phlorizinized rats may have arisen from fat. ACTH and cortisone were among the compounds having a marked effect on gluconeogenesis and cortisone was much less ketogenic than other such substances. Wells and Kendall (1940), in similar studies, had already reported a synergism between the actions of Compound E and of the thyroid gland so that the full exhibition of either was dependent upon the presence of adequate amounts of the other hormone. The similar interrelationship between cortisone and thyroxine in respect of gluconeogenesis and of fat deposition in the brown adipose tissue is striking although no link between the two processes has been shown to exist.

SUMMARY

1. Thyroxine and cortisone both promote the accumulation of fat in the brown adipose tissue of intact rats and their actions appear to be synergistic. Little or no change occurred in the water and fat-free dry matter contents.
2. In adrenalectomized animals, the response to thyroid hormone was minimal while the action of cortisone administered alone or in conjunction with thyroxine was comparable to that obtained in intact rats.
3. The administration of DCA caused no change in the size of the brown fat.
4. ACTH caused a slight hypertrophy of the brown adipose tissue, characterized by increases in all three fractions studied.

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A SODIUM-EXCRETING EFFECT OF DESOXYCORTICOSTERONE IN ADRENALECTOMIZED MICE*

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METHODS recently reported for the bioassay of desoxycortisosterone (DOC) (Dorfman, 1947; Dorfman, 1949; Deming and Luetscher, 1950; Spencer, 1950; Simpson and Tait, 1950, 1952: Luetscher and Demng, 1951; Marcus, Romanoff and Pincus, 1952) have utilized the action of DOC in adrenalectomized animals in decreasing urinary excretion of sodium, in increasing the urinary excretion of potassium or in effecting a combination of these actions. Little quantitative information has been published regarding the optimal sodium loading of the animals used for bioassay. Workers employing isotopic techniques have favored a low sodium load (Dorfman, 1947; Simpson and Tait, 1952) while those using the alteration in the urinary excretion of sodium as determined chemically or by flame photometer have favored a high salt load (Luetscher and Deming, 1951; Spencer, 1950; Marcus, Romanoff and Pincus, 1952). Although much is known concerning the effect of water loading in adrenalectomized animals (Gaunt, 1944, 1950; Hays and Mathieson, 1945; Birnie, Eversole and Gaunt, 1948; Frost and Talmage, 1951) few of these results can be applied to determining the optimal water load for the bioassay of salt retaining steroids, because urinary sodium excretion was not adequately measured.

The present studies were initiated to define more precisely the optimal sodium and water loads in adrenalectomized mice used for the bioassay of DOC. During the course of the sodium loading experiments, DOC was found to have a sodium excreting effect in small doses, and the usual sodium retaining effect in larger doses. Strain differences in sensitivity to DOC were noted in respect to both sodium excretion and sodium retention.

METHODS

Method 1

Male mice, strain CF1 Carworth Farms and strain C57 BBF1 Roscoe B. Jackson

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Memorial Laboratory, weighing from 20 to 25 grams were adrenalectomized using a single dorsal skin incision. These mice were maintained postoperatively on 0.9% sodium chloride solution, hereafter called saline, and "Friskies" dog biscuit containing 2.8 mg. sodium and 4.8 mg. potassium per gram of biscuit. Tests were performed from the fourth to the fourteenth post-operative day. Sixteen hours before assay solid food was removed and the animals were given a 10% solution of glucose or sucrose to drink. *Desoxycorticosterone (free alcohol) dissolved in 10% ethanol was used throughout* these studies. The control mice received 0.2 ml. 10% ethanol intraperitoneally, approximately 20 minutes before the assay was begun. The appropriate dose of sodium loading solution was calculated on the basis of body weight and injected subcutaneously using a number 25, two inch needle. In addition to sodium chloride the loading solution contained 5% glucose and four m.eq. per liter potassium. Urine was forcibly expressed by manual pressure over the bladder and the animals were placed in individual 600 ml. beakers with ¼ inch wire screen platforms over the bottom of the beakers. At the end of 5 hours the animals were removed and any feces present were discarded. Urine was again forcibly expressed from the bladder into the beakers. The contents of the beakers were transferred to volumetric flasks using four washes of distilled water. The total amounts of sodium and potassium in the urine were then determined with the Weichselbaum-Varney flame photometer.

Method 2

It was noted that the physical condition of the mice was often poor using Method 1 and the mortality during assay occasionally was 10–20%. For this reason the following modification was investigated. Nineteen hours before assay solid food was omitted and a solution of 10% glucose in 0.9% saline was used for drinking. Three hours before assay the mice were given only 10% glucose to drink. During the 5 hour assay period a cube of table sugar was placed in each beaker. Strain BBF1 mice ate the sugar very well, but strain CF1 mice largely ignored it. The sugar used contained approximately 0.06 mg. of sodium and 0.01 mg. of potassium per gram. In view of the small amount of sugar contaminating the urine, this source of error can be overlooked.

RESULTS

Strain CF1 Mice, Method 1

Mice of this strain were given loading solutions containing 0.5 mg., 0.3 mg., 0.1 mg. and 0.025 mg. of sodium chloride per gram of body weight administered subcutaneously in a volume of 0.5 ml. To observe the effect of the volume of loading solution on sodium excretion and DOC sensitivity the 0.5 mg. per gram, 0.1 mg. per gm. and 0.025 mg. per gram doses of sodium chloride were also injected in a volume of 2 ml. The excretion of sodium per gram of body weight under these experimental conditions with and without DOC are presented graphically in Figure 1. The decrease of urinary sodium produced by DOC was most evident with 0.5 mg. per gram of weight in a volume of one half ml. With this load a significant decrease in sodium excretion was apparent with 10 μg. of DOC ($P = 0.007$). When the same amount of sodium chloride was given in a volume of 2 ml. a dose of 25 μg. of DOC was required to establish a significant decrease in sodium

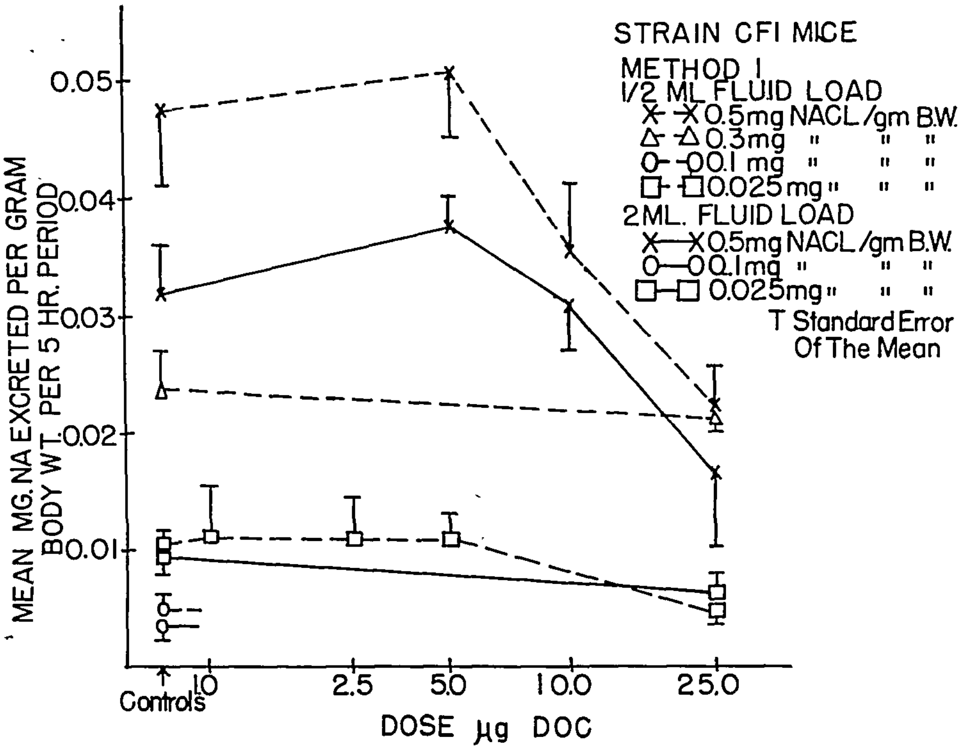

FIG. 1. The urinary sodium excretion in the strain CF1 mouse and its response to desoxycorticosterone with various fluid and NaCl loads. Standard error of the mean indicated in only one direction to avoid overlap. Each point represents the mean of from 8 to 35 animals.

excretion. With sodium loads less than 0.5 mg. of NaCl per gram of weight the control sodium excretion was correspondingly reduced. The absolute decrease in sodium excretion following DOC under these circumstances was small and too variable to be useful for assay. From these results pretreatment with 0.5 mg. NaCl per gram of body weight in 0.5 ml. was the most favorable condition for demonstrating the salt-retaining action of DOC.

Strain CF1 Mice, Method 2

Allowing adrenalectomized mice access to saline drinking water until 3 hours before assay in effect increases the salt load. The excretion of sodium with and without DOC under these conditions is presented graphically in Figure 2. Urinary sodium actually increases with doses of 5 and 10 μg. of DOC. Not until a dose of 25 μg. of DOC was given did a fall in urinary sodium from the maximum value occur. A statistical analysis of these results is presented in Table 1.

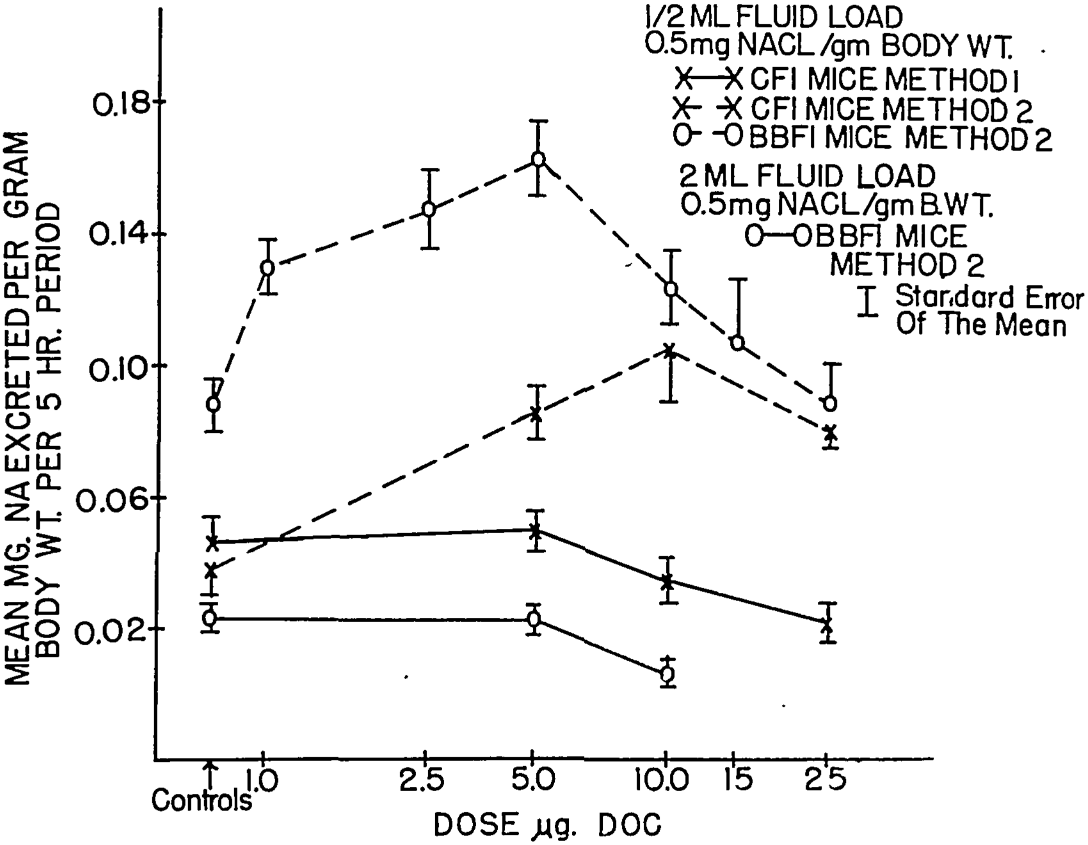

FIG. 2. The comparative effect of desoxycorticosterone on the urinary sodium excretion of strain CF1 mice and strain BBF1 mice. Standard error of the mean indicated in only one direction at times to avoid overlap. Note different scale from figure 1.

Strain BBF1 Mice

To find mice more sensitive to DOC than the CF1 strain, mice of the C57 BBF1 strain were tested. These mice tolerated sodium restriction very badly and when tested with Method 1, the mice were in such poor condition that sodium excretion was always very low regardless of fluid or sodium load used. For this reason studies on this strain are limitd to Method 2. With a sodium load of 0.5 mg. NaCl per gm. body weight and a fluid load

TABLE 1. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN CF1 MICE

	Controls	5 µg. DOC	10 µg. DOC	25 µg. DOC
No. of Animals	8	13	11	8
Mean mg. Na Excr. per gm. body wt. per 5 hrs.	0.0386	0.0857	0.1053	0.0806
Stan. error of mean	±0.0087	±0.0086	±0.0150	±0.0059

of 0.5 ml. DOC had a pronounced sodium excreting effect (Fig. 2). Maximal sodium excretion occurred with 5 µg. DOC in contrast to the strain CF1 mice which required 10 µg. DOC for maximal sodium excretion. A nearly linear fall in sodium excretion occurred when plotted against the logarithm of the dose at levels of 10 µg., 15 µg. and 25 µg. doses of DOC in the BBF1 mice. (The numerical values are analyzed in Table 2.) When the same amount of sodium chloride was given in a volume of 2 ml. (Fig. 2) no increased excretion of sodium occurred with small doses of DOC. The promotion of sodium excretion by 5 µg. of DOC decreased markedly with increasing fluid load (Fig. 3).

TABLE 2. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN BBF1 MICE

	Control	1 µg. DOC	2.5 µg.	5.0 µg.	15 µg.	15 µg.	25 µg.
No. of Animals	22	10	8	20	20	8	11
Mean mg. Na excr. per gm. body wt. per 5 hr. period	0.0884	0.1309	0.1480	0.1635	0.1256	0.1083	0.0897
Standard error of mean	±.0088	±.0083	±.0137	±.0102	±.0112	±.0192	±.0138

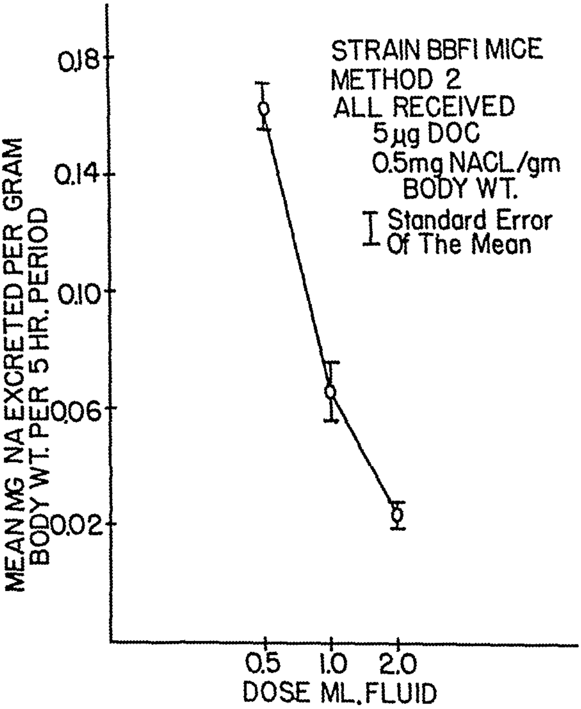

FIG. 3. The effect of increasing fluid load on the maximal urinary sodium excretion of BBF1 mice produced by 5 µg. DOC. Each point represents the mean of from 7 to 20 animals.

To minimize intestinal absorption of exogenous sodium, the drinking water containing sodium chloride was removed 5 hours rather than 3 hours before study. The control sodium excretion was somewhat lower in these experiments with a mean of 0.0601 mg. per gm. body weight per 5 hours, but increased excretion of sodium again occurred with 5 μg. DOC, the mean being 0.1006 mg. per gm. body weight per 5 hours.

Not only did the total amount of sodium but also the concentration of urinary sodium increase in strain BBF1 mice given 5 μg. DOC. Thus at the beginning of the assay 3 hours after removal of saline drinking water, the mean sodium concentration of the urine was 243 m.eq. per kilogram of urine (S.E. = ±41). Three hours after receiving 5 μg. DOC the mean sodium concentration of the urine was 424 m.eq. per kilogram of urine (S.E. = ±45). This difference is significant ($P = 0.01$). Urinary excretion of potassium was not significantly altered by doses of DOC up to 25 μg.

DISCUSSION

Speirs *et al.* (1951) were able to demonstrate that the strain of mice C57 BBF1 was about ten times more sensitive to cortisone as judged by eosinophil response than other strains tested. Our results indicate a comparable sensitivity to DOC in this same strain. These mice require 5 μg. DOC to reach maximal sodium excretion while strain CF1 mice required 10 μg. DOC for maximal sodium excretion. Significant sodium retention occurred in strain BBF1 mice with 10 μg. DOC, but strain CF1 mice required 25 μg. DOC for significant sodium retention. It would appear that mice of the C57 BBF1 strain are two to three times more sensitive to DOC than the mice of the CF1 strain.

The use of the C57 BBF1 strain for the bioassay of DOC seems possible under the conditions specified because of the wide differences in urinary sodium with doses of DOC from 1 μg. to 25 μg. The biphasic character of sodium excretion with DOC would necessitate testing several dosage levels of the material to be assayed in order to locate the value on the ascending or descending slope of the curve of urinary sodium excretion.

The promotion of sodium excretion in our experiments was unexpected. However, desoxycorticosterone has been shown to cause sodium excretion in Cushing's syndrome (Soffer, Gabrilove and Jacobs, 1949) and in normal dogs receiving high salt loads (Green, Farah and Klemperer, 1950). Large doses of desoxycorticosterone glycoside intravenously in humans with presumably intact adrenals causes sodium excretion, but with a fall in urine sodium concentration (Green *et al.*, 1950). The nearly twofold increase in control sodium excretion effected by 5 μg. DOC in adrenalectomized mice of the BBF1 strain could be largely attributed to increased urinary sodium concentration. Gastrointestinal absorption of sodium appears to contribute little to the sodium diuresis produced by DOC. Sodium excretion with 5 μg. DOC increased approximately to the same degree after animals had been off saline drinking-water for 5 hrs. Increasing the subcutaneous fluid load decreased or abolished the sodium diuresis produced by DOC.

It is known that dogs (Roemmelt, Sartorius and Pitts, 1949) and rats (Friedman, 1948; Lotspeich, 1949) maintain a normal glomerular filtration rate for a period of time after adrenalectomy when adequately hydrated. Animals used in this study had free access to saline solution and were used for a maximum period of seventeen days after adrenalectomy. It is suggested, therefore, that increasing glomerular filtration rate was probably not the mechanism whereby DOC caused increased sodium excretion in the present experiment. Adrenalectomized dogs and patients with Addison's disease have a reduced capacity to excrete sodium under a high sodium load due to increased tubular reabsorption of sodium (Roemmelt, Sartorius and Pitts, 1949; Burnett, 1951). The sodium diuresis in the present experiments would seem to be due to a depression of tubular reabsorption of sodium with small doses of DOC, thus restoring to the adrenalectomized animal a more normal ability to excrete high sodium loads. As the dose of DOC is increased the depression of tubular reabsorption of sodium with small doses of DOC is followed by the more familiar increase in tubular reabsorption of sodium. The data at hand do not permit elucidation of the mechanism of depression of tubular reabsorption of sodium by DOC.

SUMMARY

The optimal conditions for demonstrating the action of DOC in adrenalectomized mice have been studied. A sodium load of 0.5 mg. NaCl per gm. of body weight proved most satisfactory. DOC in small doses promoted sodium excretion under high sodium loads while sodium retention occurred with larger doses of DOC. Adrenalectomized mice of the BBF1 strain are two to three times more sensitive to DOC than mice of CF1 strain.

The increased sodium excretion with small doses of DOC could be accounted for by increased urine concentration of sodium. The sodium diuresis can best be explained by decreased tubular reabsorption.

ACKNOWLEDGMENTS

I would like to thank Dr. William H. Daughaday for interest in and guidance of these studies, Dr. J. Bornstein for many helpful suggestions and advice, Dr. T. E. Weichselbaum for the use of his flame photometer, Dr. N. L. Heminway, Schering Corporation, Bloomfield, N. J. for the desoxicorticosterone used in this study, and Miss S. Wood for invaluable help in the laboratory.

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EFFECTS OF ANDROGENS ON DEHYDROGENASE SYSTEMS

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WE RECENTLY reported an inhibitory effect on the succinoxidase system of rat liver homogenates by certain water-soluble androgens (Kalman, 1952). There was some evidence in this work that succinic dehydrogenase was the target of the inhibitor. Because different dehydrogenases respond similarly to a number of inhibitors it was decided to try the effect of the androgens on the malic dehydrogenase system. In addition certain observations regarding kinetics of succinoxidase inhibition are included in this report.

METHODS

Adult white rats of the Slonaker-Wistar strain were used. No sex differences in regard to the work reported were noted and males and females were used interchangeably. The animals were killed by decapitation, and appropriate amounts of liver tissue were removed, weighed, and homogenized in cold, distilled water. Homogenates were prepared as described by Potter and Elvehjem (1936). For each enzyme system control runs were made to ensure that optimal amounts of all components were present. Control QO_2 values fell within the range of conventional results. Calculations of oxygen consumption were based on time periods during which control vessels showed essentially linear oxygen uptake. The gas phase for all systems was oxygen. In addition to the 3.0 ml. of reaction mixture, 0.1 ml. of 5% KOH was placed on filter paper in the center well of each vessel.

For the malic dehydrogenase assay the method of Potter (1946) was used. The Warburg vessels contained 0.3 ml. each of 0.5 M malate, 0.5 M glutamate, 1% diphosphopyridine nucleotide (DPN) solution, 0.0004 M cytochrome C, 0.8 ml. of 0.1 M PO_4 buffer at pH 7.4, inhibitor and homogenate in amounts specified below, and distilled water to make 3.0 ml. The DPN was added from the sidearm at the start of the reaction. Nicotinamide was omitted because it did not enhance oxygen consumption over the time periods employed.

The succinoxidase system used was that described in Umbreit's text (1949). The reaction vessel contained 0.3 ml. each of 0.004 M $CaCl_2$, 0.004 M $AlCl_3$, 0.0001 M cytochrome C, 0.5 M succinate, 1.0 ml. of 0.1 M PO_4 buffer at pH 7.4, and inhibitor as described below. A 5% liver homogenate was prepared in the usual way and then centrifuged for 10 minutes at 600×gravity. Homogenate from the supernatant was added to reaction vessels which had been filled with the other reactants and chilled for several minutes.

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The androgens used were sodium androsterone sulfate, sodium testosterone sulfate, and testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). These steroids were used in aqueous solutions. The sodium testosterone sulfate and sodium androsterone sulfate are water soluble at pH 7.4. Testosterone 17-β diethylaminoethylcarbonate hydrochloride is insoluble at pH 7.4 (the pH of the reaction vessels) and above, forming a very fine suspension in an aqueous medium. This suspension may be dissolved by bringing the pH back to neutrality or to the acid side. During the course of this work some diethylaminoethanol hydrochloride was made available through the courtesy of Dr. E. Oppenheimer. This compound was tested for possible *in vitro* effect since it forms the major part of the side chain in "soluble" testosterone.

RESULTS

Inhibition of the malic dehydrogenase system by small concentrations of "soluble" testosterone is shown in Table 1. Sodium testosterone sulfate proved to be much less effective an inhibitor as indicated by the concentrations required. Sodium androsterone sulfate was not available for test on the malic dehydrogenase system.

TABLE 1. EFFECT OF WATER SOLUBLE ANDROGENS ON THE MALIC DEHYDROGENASE SYSTEM OF RAT LIVER

(QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.)

Homogenate mg. dry weight per vessel	*Molar concentration*		QO_2	Per cent inhibition
	Sodium testosterone sulfate	"Soluble" testosterone		
1.5	—		98	Control
1.5	3.4×10^{-4}		82	16.3
1.5	6.8×10^{-4}		46.8	52.3
1.5	1.0×10^{-3}		26.0	75.0
1.5	1.4×10^{-3}		14.0	86.0
1.5	1.7×10^{-3}		16.0	84.0
3.0		—	105	Control
3.0		1.8×10^{-5}	100	4.8
3.0		2.7×10^{-5}	90	14.2
3.0		3.6×10^{-5}	46	56.0
3.0		4.5×10^{-5}	23	78.0
3.0		5.4×10^{-5}	21	80.0
3.0		6.3×10^{-5}	17	84.0
3.0		7.2×10^{-5}	12	88.5

In Table 2 data are presented which show inhibition of the succinoxidase system by "soluble" testosterone, by sodium androsterone sulfate, and by sodium testosterone sulfate. The inhibitory effect of these compounds as shown by the concentrations used was most pronounced with "soluble" testosterone, less with sodium androsterone sulfate, and least with sodium testosterone sulfate. Diethylaminoethanol hydrochloride does not inhibit succinoxidase in concentrations of 3.5×10^{-5} molar. Such amounts are equivalent to the amount of this compound which is present in the side chain of "soluble" testosterone at concentrations of 9×10^{-5} molar.

TABLE 2. EFFECT OF WATER SOLUBLE ANDROGENS ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER

QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.
All vessels contained 3.0 mg. dry weight of homogenate.
Concentrations of drugs are expressed in final molar concentration

Sodium testosterone sulfate	QO_2	Per cent inhibition	Sodium androsterone sulfate	QO_2	Per cent inhibition
—	76.0	Control	—	86	Control
3.4×10^{-4}	45.0	41.0	9×10^{-5}	81.5	18.9
6.8×10^{-4}	32.0	58.0	1.3×10^{-4}	68.0	26.7
1.0×10^{-3}	13.8	81.5	1.8×10^{-4}	61.0	27.8
1.4×10^{-3}	4.0	95.0	2.3×10^{-4}	60	33.4
1.7×10^{-3}	5.0	94.0	2.7×10^{-4}	57.4	62.3
2.0×10^{-3}	2.6	96.0	3.2×10^{-4}	34.7	92
			3.6×10^{-4}	0	100
"Soluble" testosterone	QO_2	Per cent inhibition	diethylamino-ethanol · HCl	QO_2	Per cent inhibition
—	74	Control	—	87	Control
9×10^{-6}	66	10.8	3.5×10^{-5}	91	—
1.8×10^{-5}	36	51.5	3.5×10^{-5}	94	—
2.7×10^{-5}	14	81.0			
3.6×10^{-5}	8	89.0			
4.5×10^{-5}	3	96.0			
5.4×10^{-5}	2	97.5			

Preliminary results suggest that the water-soluble androgens also have an inhibitory effect on the lactic dehydrogenase system.

The curves of Figure 1 were derived from the data in Table 1 by plotting per cent inhibition of oxygen consumption by "soluble" testosterone for both malic and succinic dehydrogenase systems. Similar curves are obtained for inhibition by the sodium salt of testosterone sulfate of the malic dehydrogenase system and for the inhibition by sodium androsterone sulfate of the succinoxidase system. The inhibition of succinoxidase by sodium testosterone sulfate yields an atypical curve, no explanation for which is at hand.

Incubation time had no apparent effect on the inhibition of succinoxidase in liver homogenate by "soluble" testosterone in an experiment in which succinate was added from the side arms into the main chamber of the Warburg vessels at successive time intervals.

Figure 2 shows the effect of added homogenate on the testosterone inhibition of succinoxidase. Data are plotted according to Ackermann and Potter (1949). At each inhibitor concentration the same amount of enzyme is "bound" regardless of the amount of homogenate present.

DISCUSSION

The most interesting observation noted was the inhibition of the malic dehydrogenase system by "soluble" testosterone and by sodium testosterone sulfate. Erway *et al.* (1947) have reported that sodium androsterone

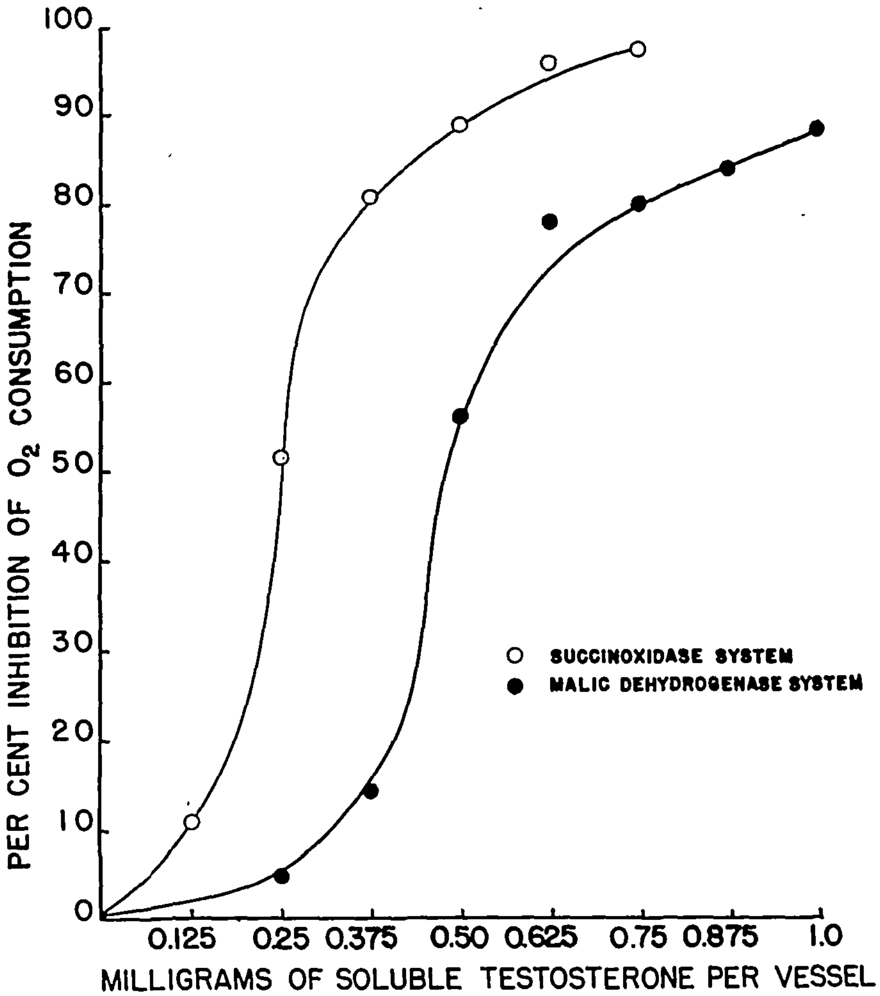

FIG. 1. The effect of concentration of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone) on the malic and succinic dehydrogenase systems. 0.125 mg. "soluble" testosterone is equivalent to 9×10^{-6} molar concentration.

sulfate has no significant effect on this enzyme system. They used inhibitor concentrations of 10^{-4} molar.

The inhibition of the succinoxidase and malic dehydrogenase systems by water-soluble steroids used appeared not to fit classical formulations (Lineweaver and Burk 1932). However, the data are compatible with the "irreversible" type of inhibitor described by Ackermann and Potter (1949). A definite reduction in oxygen consumption is effected by a given increment of inhibitor and is not reversed by increasing the amount of enzyme present (Fig. 2). Bain (1949) has demonstrated one instance of this type of inhibition in the effect of certain phosphate esters (diisopropylfluorophosphate, tetraethylpyrophosphate) on cholinesterase. Hayano and Dorfman (1951) showed a similar relationship for the inhibition of a d-amino acid oxidase by desoxycorticosterone.

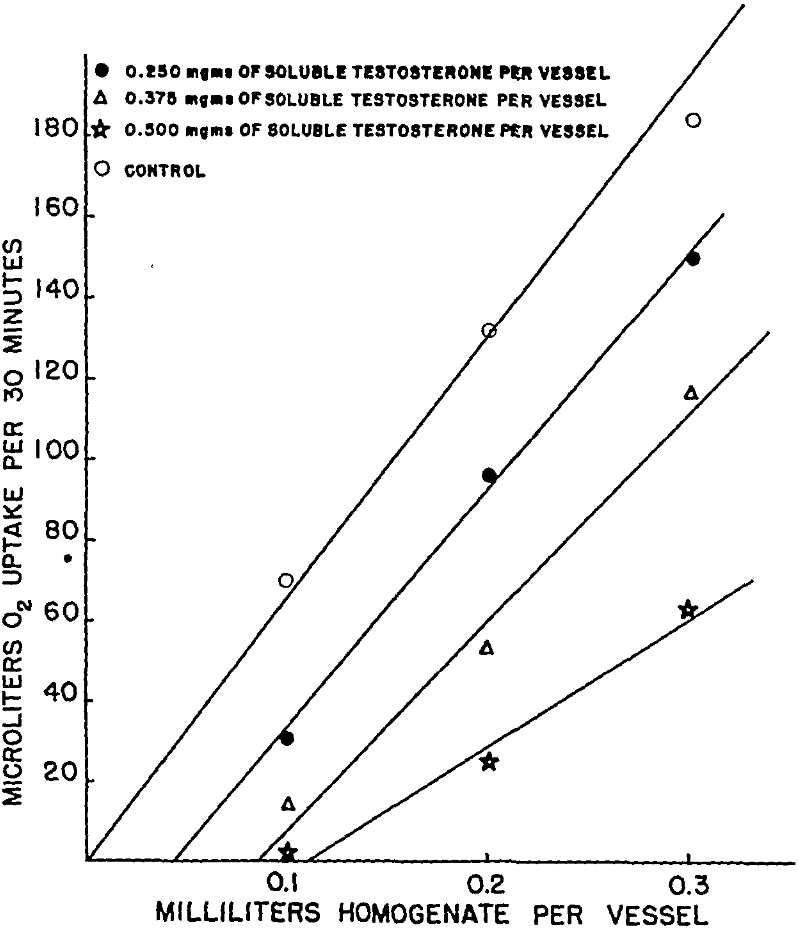

FIG. 2. The effect of increasing amounts of homogenate on the inhibition of the succinoxidase system by different amounts of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). 0.250 mg. soluble testosterone is equivalent to 1.8×10^{-5} molar concentration.

Dr. D. M. Greenberg suggested that the sigmoid shape of the curves relating per cent inhibition to inhibitor concentration (Fig. 1) resembles an adsorption isotherm. Such curves have been obtained, for example, for the amount of water vapor adsorbed on charcoal under conditions of varying pressure (Coolidge 1937). If we consider per cent inhibition analogous to inhibitor uptake, and inhibitor concentration analogous to "pressure," we may postulate that the steroid is adsorbed by the enzyme. The inhibition of saccharase by certain basic dyes has been described by Quastel and Yates (1936). When per cent inhibition was plotted against pH sigmoid curves similar to the ones plotted here were obtained. They suggested that the anion form of saccharase was better able to combine with basic dyes.

The significance of the variation in water solubility of the androgens mentioned above is not apparent. It may be that the long side chain of "soluble" testosterone makes this compound more susceptible to adsorption phenomena.

The site of action of the androgens on the dehydrogenase systems is thought to be the dehydrogenases themselves. Evidence for this has accumulated mainly by inference after other hydrogen carriers were bypassed or proved to be less susceptible to the inhibitors (Eisenberg *et al.*, 1949). A promising method of approach would seem to be fractionation of the components of the Warburg reaction mixtures in an attempt to recover the enzyme-inhibitor complex. Such an approach might also be used to study the enzyme-inhibitor "binding."

SUMMARY

The effects of water-soluble androgens on malic dehydrogenase and succinoxidase of rat liver homogenates were studied using conventional Warburg techniques. These substances were found to inhibit both enzyme systems in a manner suggesting an irreversible combination of enzyme and inhibitor. The data were also suggestive of some type of adsorption phenomenon involving enzyme and steroid.

Acknowledgements

The steroids used were kindly supplied by Dr. E. Oppenheimer of Ciba Pharmaceuticals.

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PATHWAYS OF ELIMINATION OF C^{14}-LABELED THYROXINE IN THE RAT[1]

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INTRODUCTION

TAUROG, Briggs, and Chaikoff (1951) using I^{131}-L-thyroxine demonstrated the presence of an unknown metabolic product, in addition to thyroxine, in the bile of rats. These investigators (1952) then reported that the thyroxine-containing derivative may be a glucuronide, but not a peptide. They further concluded that the conjugate is the primary metabolic product of endogenous thyroxine, and that the bile is the chief pathway of elimination for this hormone.

The present study is concerned with the elimination of DL-thyroxine-1-C^{14} in the bile, urine, and expired air. Further analysis of the bile has indicated that the thyroxine conjugate is not a peptide.

METHODS

DL-thyroxine-1-C^{14} used was that synthesized by Wang, Hummel, and Winnick (1952).[4] Its specific activity was 5.3 μc per mg. In all radioactivity measurements, the observed counts per minute were converted into equivalent μgm. of thyroxine, by reference to the known specific activity of the administered C^{14}-thyroxine. Samples were generally counted to within a probable error of 2 or 3%, and the values corrected for self-absorption. By way of illustration, 0.1 ml. of bile had of the order of 300 counts per minute, or 20 times background, with our counter.

Adult male Sprague-Dawley rats, fed ad libitum, were employed. The bile duct was cannulated with polyethylene tubing (inside diameter, 0.011 inch). When a good rate of flow was observed, the abdomen was closed, and the animal suspended in a restraining harness. Thyroxine was injected subcutaneously at a dose level of 780 μgm. (one μM) per kilo body weight. Bile samples were collected in 2 ml. portions over a 12 to 14-hour

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[4] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U. S. Atomic Energy Commission.

period. The radioactivity of the whole bile was determined by drying 0.1 ml. aliquots on aluminum plates, and counted in a thin mica window Geiger counter.

Paper chromatograms were performed as described by Taurog and co-workers (1951). Aliquots of 0.1 ml. bile were chromatographed immediately after the bile was collected. After the chromatogram was completed, the paper was cut into 1 cm. strips, and each strip extracted three times with 0.5 ml. of 0.01N NaOH. The extracts were air dried on aluminum plates for counting. Approximately 53% of the added thyroxine activity was recovered from the paper chromatograms.

The whole bile was subjected to the Van Slyke ninhydrin-carbon dioxide method (1941) which indicates the presence of free carboxyl and amino groups in amino acids. These determinations were performed at pH 2.5 on 0.1 ml. bile samples. The $BaCO_3$ was collected and counted as described by Winnick (1950).

A Benedict closed circuit metabolic apparatus was employed for the collection of the expired $C^{14}O_2$. The latter was removed from the expired air by bubbling the circulating gases through a 1N NaOH solution which was replaced at 2 or 3-hour intervals. Aliquots of the NaOH solutions were treated with excess $BaCl_2$, the precipitated $BaCO_3$ washed, dried, and plated for counting.

The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples, obtained at the various time intervals. The urine, which was collected from rats placed in metabolic cages, was plated as described for the bile.

RESULTS AND DISCUSSION

Figure 1 shows that the C^{14} of carboxyl-labeled DL-thyroxine is most rapidly eliminated by way of the bile. About 20% of the radioactivity was found in the bile in 12 hours. Almost identical results were obtained in 5 repeated experiments. Taurog and co-workers (1951) give a comparable value of 28% eliminated in 12 hours, for a subcutaneous injection of 330 μgm. of I^{131}-labeled L-thyroxine per kilo.

The appearance of 10% of the C^{14} in respiratory carbon dioxide in 12 hours indicates that the alanine portion of the thyroxine molecule is catab-

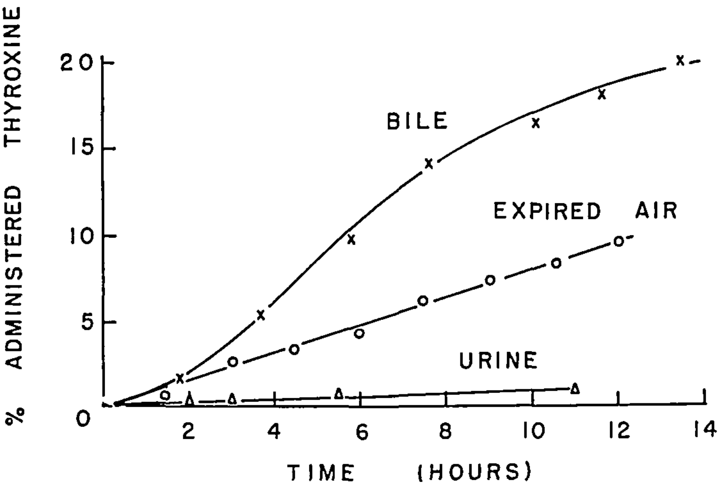

FIG. 1. Elimination of C^{14}-carboxyl labeled thyroxine by different routes.

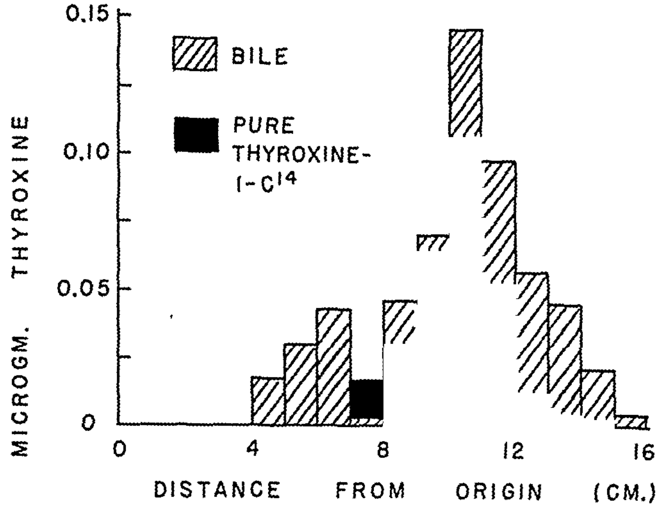

FIG. 2. Chromatogram of 0.1 ml. aliquot of a 2 ml. sample of bile, taken 1.8 to 3.8 hours after administering thyroxine-1-C^{14}.

olized. Essentially the same rate of elimination of $C^{14}O_2$ was obtained in 5 other experiments.

A relatively low C^{14} concentration, about 1% of the total dose, appeared in the urine in 12 hours. The average value was 1.2% for 10 such experiments. This radioactivity may represent, in part, urea derived from $C^{14}O_2$ by way of the ornithine cycle. Gross and Leblond (1949) reported a urinary excretion of about 10% of intravenously administered I^{131}-thyroxine in rats after 24 hours. Most of the I^{131} could not be extracted from the urine by butanol, and was referred to as "non-thyroxine" iodine.

The results of a paper chromatogram performed on bile from a rat given C^{14}-thyroxine is shown in Figure 2. The small peak accounted for 10%, and the larger, more rapidly moving peak 53%, of the total C^{14} in the bile sample. The position of the larger peak is identical with that of thyroxine. These results show that, at the 780 μgm./kilo dosage, free thyroxine predominates in the bile. This conclusion is in accord with the finding of Taurog and co-workers (1952) that large doses of thyroxine-I^{131} are conjugated to only a very limited extent in the rat. Because of the limitations imposed by the specific radioactivity of our thyroxine-1-C^{14}, it was not possible to employ the latter at physiological levels.

The data in Figure 3 indicate that virtually all of the C^{14} of the bile is released by ninhydrin treatment. The agreements are within the limits of experimental error. Van Slyke, Dillon, MacFadyen, and Hamilton (1941) have shown that amino acids are decarboxylated by ninhydrin only when both amino and carboxyl groups are in the uncombined state. Although

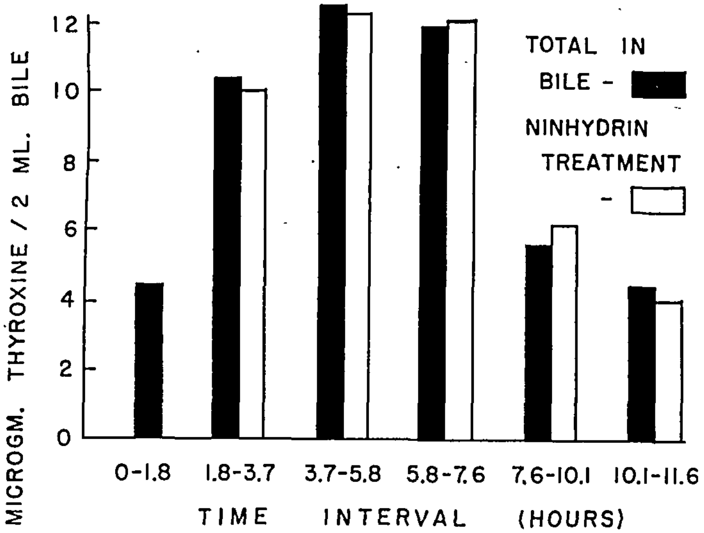

FIG. 3. Thyroxine excretion, as measured by total C^{14} in bile, and by $C^{14}O_2$ released from bile by ninhydrin.

chromatography indicates that a minor portion of the thyroxine exists in conjugated form, this fraction may be bound through the phenolic hydroxyl group, and hence would still react with ninhydrin.

SUMMARY

The various routes of elimination of thyroxine-1-C^{14} in normal adult rats were investigated. Labeled thyroxine was administered subcutaneously at a level of 780 μgm./kilo. The bile was the major route of excretion, and contained about 20% of the C^{14} after 12 hours. Approximately 10% of the C^{14} appeared in the expired CO_2, and 1% in the urine, in this period.

Paper chromatographic analysis of a bile sample taken between 2 and 4 hours, revealed the presence of a small secondary component, in addition to a large thyroxine peak. Treatment of bile samples with ninhydrin resulted in a quantitative release of C^{14} as $C^{14}O_2$. This finding indicates that both amino and carboxyl groups of the thyroxine in the bile are in an uncombined state.

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ESTIMATED DURATION OF THE SPONTANEOUS ACTIVATION WHICH CAUSES RELEASE OF OVULATING HORMONE FROM THE RAT HYPOPHYSIS[1]

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INTRODUCTION

NEUROGENIC activation of the rabbit adenohypophysis causing discharge of ovulating hormone is now considered to be accomplished within about one minute after coitus (Sawyer, Markee *et al.*, 1947, 1949). Actual discharge into the bloodstream of hormone sufficient for ovulation requires approximately one hour in that species (Fee and Parkes, 1929).

Whereas, in spontaneously ovulating animals, discharge of the hormone was long thought to be invoked by direct action of ovarian steroids on the hypophysis, there is now strong evidence that this effect is mediated by the hypothalamus (Sawyer, Everett and Markee, 1949; Everett and Sawyer, 1949). Activation of the hypophysis in cyclic rats occurs within a restricted period of time during the day of proestrus (Everett, Sawyer and Markee, 1949). In our colony this critical period is between 2 and 4 P.M. for the great majority of animals (4-day cycle), about 9 to 11 hours before ovulation. The injection of certain blocking agents at 2 P.M. or earlier will prevent ovulation, while their injection at 4 P.M. will not usually interfere with it.

Studies with the rabbit have demonstrated that antiadrenergic agents of the β-haloalkylamine series (Dibenamine and SKF-501) and certain anticholinergic agents (atropine and Banthine) will block the reflexogenous stimulus to the hypophysis when injected intravenously within a fraction of a minute after coitus (Sawyer, Markee *et al.*, 1947, 1949, 1950, 1951). These agents are similarly effective in blocking the "spontaneous" activation of the hypophysis in cyclic rats. In addition, the barbiturates have been effective in the latter species, apparently because they can be intro-

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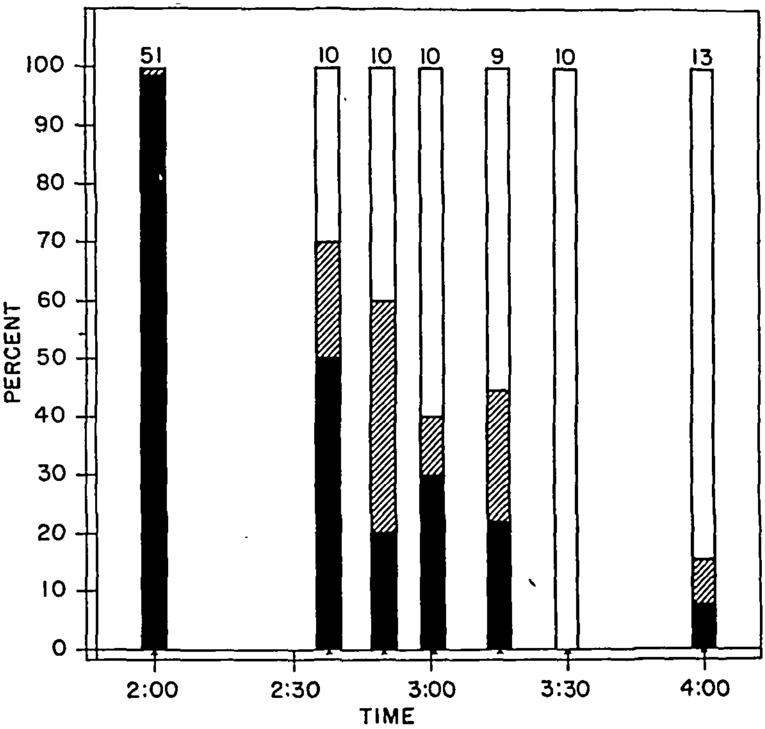

FIG. 1. Results of injection of atropine on the day of proestrus at different times between 2:00 and 4:00 P.M. Solid black: complete blockade. Cross-hatching: partial blockade. White: complete ovulation. The number above each bar indicates the respective number of rats in that time-group. The 2:00 and 4:00 P.M. data are from previous work with atropine and Nembutal (From Everett, 1952, by courtesy of the Ciba Foundation).

gression, for the following considerations. During the first 50 minutes 65% of 20 rats had begun activation, while during the succeeding 40 minutes the percentage increased to only 83% of the remaining 29 rats. Even as late as 4 P.M. 1 case of complete blockade and 1 of partial blockade were encountered. While the former may have been a rare animal which whould have failed to ovulate without atropine, the latter was not. In her case activation must have begun only a short time before injection. It seems probable that an average of the combined 3:30 and 4 P.M. data gives a more nearly true value than either alone.

The occurrence of 9 cases of partial activation, rather uniformly distributed among the 2:40–3:15 time-groups, confirms the earlier impression that the activation stimulus is not momentary. Such a conclusion, however, requires that under the conditions of administration of atropine in massive doses by the subcutaneous route the drug abruptly assumes full blocking concentration at its locus of action, with no significantly prolonged interval during which it would only moderately diminish the strength of stimulus. The following control experiments seem to give this assurance.

A threshold dose for subcutaneous injection was arrived at empirically: 230 mg./kg. (23 mg. atropine sulphate per ml. of physiological saline). This amount was administered to each of 15 proestrous 4-day cyclic rats at 2:00 P.M. At autopsy next morning the tubes were searched for ova and the excised ovaries were critically examined under the dissecting microscope. In the absence of tubal ova all follicles showing any degree of hyperemia were dissected out with iridectomy scissors and were individually crushed in a small drop of saline between slide and coverslip for microscopic examination. Eight of the 15 rats were judged to have been completely blocked, while 6 rats had ovulated completely. In the remaining animal 3 follicles were markedly hyperemic and when these were crushed prominent corona radiata were seen. Maturation of the ova was in progress and the first polar body was seen in one instance. Partial activation in this one exceptional animal may be assigned to an unusually early beginning of hypophyseal activation, similar to the one case from the Nembutal series included in the 2:00 P.M. group in figure 1. The absence of *numerous* examples of partial activation in the present series, intergrading between complete blockade and complete ovulation, attests an essentially all-or-none effect of atropine at threshold level and signifies that no prolonged interval of partial effectiveness of atropine precedes the time at which full blockade is accomplished.

The second series of control experiments approached the question in another way. If the cases of partial blockade in the definitive series (Fig. 1) were mainly representative of a prolonged interval of partial effectiveness of atropine associated with slow absorption from the subcutaneous site, the intravenous injection of the maximum amount tolerated, immediately before the standard subcutaneous dose at some time during the critical period, should have an all-or-none effect. Most female rats of this strain will tolerate intravenous injection of 70 mg./kg. of atropine sulphate (35 mg./ml. in Ringer-Locke solution). This amount was now given to each of 10 proestrous 4-day cyclic rats at one or another time between 2:30 and 3:20 P.M., the entire amount being introduced within 70 to 80 seconds. (Two additional rats died within a few minutes after injection.) Subcutaneous injection of the 700 mg./kg. dose was completed within a minute afterward. Marked tremors were noted before the intravenous injections were complete and involuntary contractions of various muscle groups were regularly seen when the animal was released. It is significant that in 3 of the 10 rats partial blockade was encountered. At autopsy on the morning after injection, 2 of these had a single follicle showing corona formation and maturation changes, while the third animal had shed 2 ova and most of her unruptured follicles were markedly hyperemic. This considerable number of animals showing partial effects supports the interpretation that they arise from abrupt interruption of a lengthy process of hypophyseal activation.

Inasmuch as the chance of interrupting a progressive process is a direct function of its duration, the latter can be estimated in the present instance from the percentage partially activated. If regression of percentage blocked were linear with respect to time, a direct transformation could be made from percentage partially activated to minutes elapsed. Inasmuch as the regression is curvilinear, however, certain approximations are required to allow such transformation.

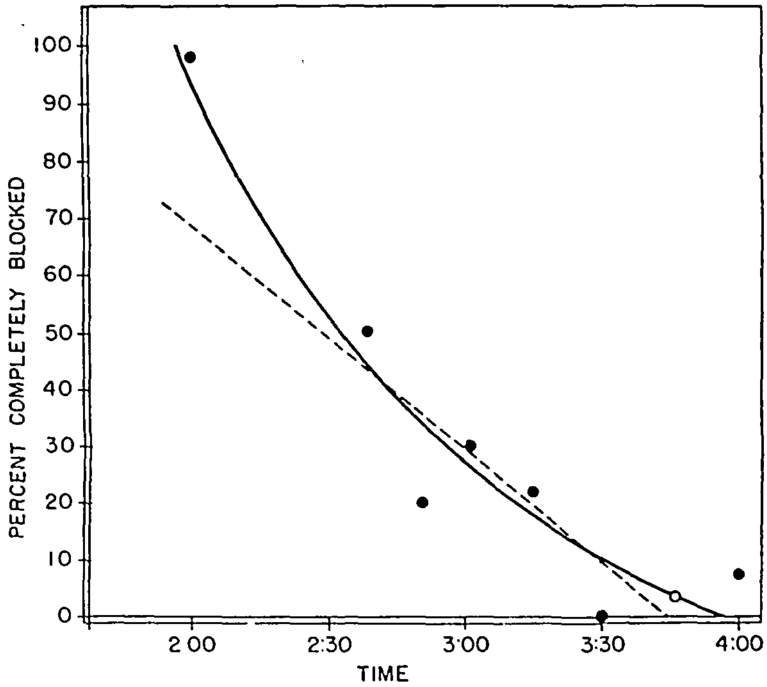

FIG. 2. Diminishing frequency of complete blockade after 2:00 P.M., inversely measuring the beginning of hypophyseal activation. The logarithmic curve is mathematically fitted to the percentages represented by solid circles (the open circle represents the average of the 3:30 and 4:00 P.M. values). The straight (broken) line is approximated to the curve between the 2:40 and 3:30 points (see text).

In Figure 2 a logarithmic curve is mathematically fitted to the points representing percentages completely blocked. A straight (broken) line is approximated to the logarithmic curve between the 2:40 and 3:30 points. The slope of this line is such that 10% on the y axis corresponds to 15.3 minutes on the x axis. In terms of this slope, the 9 cases (18.4%) of partial activation among 49 rats of the 2:40–3:30 groups correspond to 28 minutes on the time axis.

This value, however, is admittedly a very rough approximation and has little significance in itself. An important source of error is chance variation in the percentage partially activated. By use of Fisher's table $VIII_1$ (Fisher and Yates, 1949), entered with $a = 9$ ($a/N = 9/49 = 0.184$) the lower limits of expectation were obtained.[3] At probability levels of 0.025 and 0.005 these limits were 10% and 8%, respectively, corresponding to 13 and 10 minutes (fig. 3). Thus, the probability is high (0.975) that duration of stimulation is greater than 13 minutes, based on the slope of the straight line from figure 2. An extremely unlikely lower limit may be calculated by disregarding the evident curvilinearity of regression and employing as the steepest plausible slope a straight line drawn between the 2:40 P.M. and 3:30 P.M. values. Since on this assumption all activation would be completed before 3:30, this group is excluded in calculating the percentage partially activated and Fisher's table $VIII_1$ is entered with $a = 9$ ($a/N = 9/39 = 0.231$). At the probability level of 0.005 this gives a lower limit of 9 minutes (Fig. 3, lower dotted line).

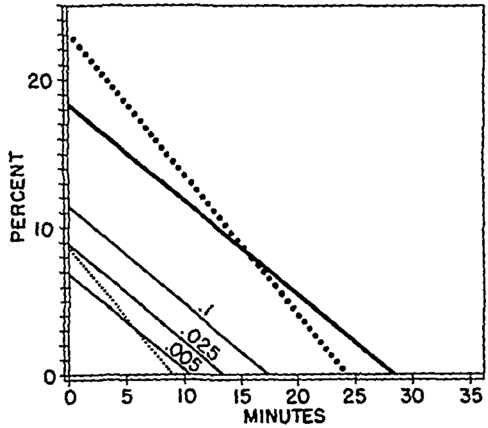

FIG. 3. Relation between percentage partially blocked and time. The heavy solid line has the same slope as the broken line in figure 2. Lower limits of expectation at three levels of probability are represented by the narrow solid lines. Significance of the *dotted lines is given in the text.*

The calculated limits do not take into consideration the numerous cases of partial activation in other comparable experiments, e.g. the progesterone-atropine series cited earlier (Everett and Sawyer, 1949). Among 78 appropriate examples from such experiments, 20 (24%) were classed as partial activation. Thus the frequency of partial activation in the present series appears to be more truly representative than the calculations imply. Consequently it seems reasonable to judge that in any given rat the duration of stimulus is within the range of 20 to 35 minutes.

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[3] The upper limit was given directly by the fact that 3 of 10 rats in the 2:40 group were fully activated. Their periods of activation must have been less than 40 minutes.

DISCUSSION

The results confirm the earlier conclusion (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950) that in 4-day cyclic rats of our colony a specific neurogenic stimulus, essential to the discharge of ovulating hormone, passes to the adenohypophysis within restricted, predictable time limits on the afternoon of the day of proestrus. In addition, it is now shown that this stimulus in any one individual is considerably shorter than the 2-hour critical period (2 to 4 P.M.) previously defined. On the other hand, the stimulus lasts much longer than the reflexogenous one in the rabbit. In that species the cholinergic (atropine-sensitive) phase is thought to be completed early in the first minute post-coitum, while the adrenergic (Dibenamine-sensitive) phase is only slightly longer, approximately 1 minute (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949; Sawyer, Markee and Everett, 1950). In the rat the cholinergic phase of the stimulation process lasts for over 9 minutes, more probably between 20 and 35 minutes and certainly less than 40 minutes.

It seems likely that in the rat the central (Nembutal-sensitive) phase and the cholinergic and adrenergic phases of the stimulation process take place almost concurrently. Time differences of the order found in rabbits would be obscured by the far greater over-all duration.

A quantitative relationship apparently exists between length of time of hypophyseal stimulation and the amount of ovulating hormone released. Such an interpretation rests on the finding in rabbits that none of the blocking agents interferes directly with passage of ovulating hormone from the hypophyseal cells into circulation (Sawyer, *et al.*, loc. cit.). Since a species difference in this respect is unlikely, the numerous cases of partial blockade in the present series should then represent incomplete release resulting from foreshortened stimulation. Indeed, if the normal period of stimulation approximates half an hour, stimulation and release may proceed concurrently. While in rabbits about an hour is required for release (Fee and Parkes, 1929), the time in rats could easily be somewhat shorter.

It should be noted that in at least two of the species in which ovulation depends on copulation, relatively prolonged stimulation from lengthy or repeated copulation is indicated (*ferret,* Hill and Parkes, 1932; *mole shrew,* Pearson, 1944). In the mole shrew 1 to 3 copulations per day are said to be ineffective, whereas ovulation regularly follows when the number is increased to 10 to 15 for another two days. In contrasting the rabbit and the ferret, Hill and Parkes spoke of the "trigger" mechanism characteristic of the former species, although its momentary nature had not been elucidated at that time. It seems probable that in spontaneous ovulators and reflex ovulators alike there may be rather wide species variation in the duration of the stimulation process.

There are some indications that under normal circumstances the rat hypophysis continues to be stimulated for a considerably longer period than that which is just sufficient to release the minimal amount of gonadotrophin capable of ovulating all the follicles. Everett, Sawyer and Markee (1949) noted in the original 4 P.M.-atropine series that while all animals ovulated, the corpora lutea from the preceding cycle failed to store visible cholesterol within the usual time. Such cholesterol storage has been shown to be a function of LH (Everett, 1947). Another function of LH is seen in the depletion of cholesterol in the interstitial tissue (Claesson and Hillarp, 1947; Everett, 1949). In our 4-day cyclic rats such depletion is normally so extreme on the morning after ovulation that the ovarian interstitium presents a dull, watery appearance. In the present experiments this extreme depletion was noted in only 24 of 35 rats (69%) in the "fully ovulated" category. It seems likely, therefore, that the estimate of 20 to 35 minutes simply represents the minimal stimulation necessary for ovulation and that the normal stimulation lasts for some longer time, causing relase of an excess of gonadotrophin. This factor of insurance may be reflected not only in the lipid changes in corpora lutea and interstitial tissue, but also in the uniformity of ovulation time observed in these animals.

SUMMARY

Previous study of 4-day cyclic rats (inbred Vanderbilt strain) has defined a 2-hour interval during proestrus, within which neurogenic ovulatory activation of the adenohypophysis takes place. In the present study, 49 such rats were injected with the standard blocking dose of atropine at different times during that interval and were autopsied the next morning. From the results, in particular from the relative frequency of partial blockade, it is estimated that to release sufficient gonadotrophin for complete ovulation the required duration of stimulus is between 20 and 35 minutes. Collateral evidence suggests that the stimulus may normally continue somewhat longer, serving to release an excess of ovulating hormone.

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ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT AT VARIOUS AGES[1,2]

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NUMEROUS investigations have been made of the pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) concentrations of various tissues and organs in different experimental animals. These have been summarized in tabular form by Davidson (1947, 1950), Schneider (1946) and Schneider and Klug (1946).

Cytochemical studies covering the content and distribution of nucleic acids in many tissues and organs have been reviewed by Davidson (1950), Brachet (1950) and Caspersson (1950).

Examination of the literature has shown that little attention has been given to the concentration and distribution of these important substances in the testis. McCarter and Stelges (1948) in an examination of methods for the determination of phosphorus-containing constituents in rat tissues have included a limited survey of organic phosphorus compounds in the testis. Using cytochemical techniques Brachet (1933, 1940, 1941) has studied insect and amphibian testes and Caspersson (1933, 1936) grasshopper testes.

The paucity of information concerning the nucleic acids of the testis, particularly the mammalian testis, has suggested the desirability of such a study in the rat by chemical and cytochemical techniques. In order to provide a more complete survey of other phosphorus-containing substances additional studies were made.

Acid-soluble phosphorus (ASP) which according to Lipman (1941) and

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[2] This paper is a part of the content of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy of the Graduate College of the State University of Iowa, February 1952.

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Kalcar (1941) represents primarily intermediates and enzymes in carbohydrate and energy metabolism, was determined because of the reputed relationships between these and nucleic acid metabolism (Hevesy, 1948; Davidson, 1950). The lipid phosphorus (LP) fraction was studied since Claude (1939, 1940, 1941, 1949) has demonstrated a relationship between cytoplasmic pentose nucleic acid and phospholipid.

The present report, therefore, includes chemical data on the four phosphorus fractions and cytochemical data on the nucleic acids in the rat testis at different ages. Similar data for testes with experimentally induced impairments in spermatogenesis are being reported in a separate paper (McEnery and Nelson, 1952).

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory were used throughout the study. Animals were sacrificed when ten, twenty, thirty, forty, sixty and one hundred eighty days of age and the testes removed for chemical analysis and for cytochemical and routine histological preparations.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). In the case of each procedure duplicate determinations were made on testicular tissue from each animal except in the ten day series where the two testes from each of five animals were pooled. All were wet weight determinations and moisture contents were checked in each group.

Cytochemical Preparations

Portions of the testes were fixed in Zenker acetic fluid for one hour and fifteen minutes and prepared according to the Feulgen technique (Feulgen and Rossenbeck, 1924) for DNA and the methyl green pyronin technique for PNA and DNA (Brachet 1940, 1941). Since the latter technique is extremely capricious and for specificity should be accompanied by ribonuclease digestion of control sections the cytoplasmic basophilia demonstrated by it was used only as a rough index of PNA. It may be mentioned, however, that Taft (1951) in a recent paper offers substantial evidence for the specificity of the methyl green pyronin reaction as an indicator of the presence of both PNA and DNA.

Cytological Preparations

Portions of the testes were fixed in Bouin's fluid and stained with hematoxylin and eosin, Masson's and Mallory-Azan procedures.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentrations of phosphorus in the PNA, DNA, TNA, ASP and LP fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the differences between the means of the values obtained for these fractions in the animals of the younger age groups as compared with the equivalent values in adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2.

It is readily seen that as the age of the animals progresses toward maturity the testicular concentration of each of the phosphorus fractions decreases with the exception of the lipid fraction which remains approximately constant (Fig. 1). Conversely in terms of absolute amounts there is a progressive increase in each of the phosphorus fractions with increase in age (Fig. 2).[4]

Table 1. The mean concentrations of the five P fractions and the calculated mean concentration of PNA and DNA for each age expressed as mg. per 100 gm. of fresh tissue

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	120.01 ±6.459* 0.001†	25.81 ±0.964 0.001	141.32 +2.632 0.001	148.79 ±2.454 0.001	34.13 ±1.718 0.20	1272.10	260.68
20 day	22	110.84 ±0.417 0.001	26.67 ±0.842 0.001	137.85 ±2.213 0.001	126.98 ±1.707 0.001	37.39 ±0.861 0.01	1174.90	269.36
30 day	21	86.63 ±1.546 0.001	20.83 ±0.562 0.001	107.35 ±1.765 0.001	94.72 ±2.038 0.001	32.70 ±2.071 0.20	918.27	210.38
40 day	22	70.31 ±1.997 0.001	13.86 ±0.521 0.001	86.60 ±1.675 0.001	73.55 ±2.115 0.001	33.76 ±1.191 0.20	748.28	139.98
60 day	20	59.45 ±1.794 0.20	12.21 ±2.552 0.20	73.94 ±1.862 0.20	67.67 ±2.119 0.20	34.76 ±1.113 0.20	630.17	123.32
Normal Adult (180 day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.39	124.23

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

Total phosphorus determinations were made on five samples from normal adult testes and the mean values of these were compared with the sums of the mean values for the four fractions in the same testes. The total phosphorus content was greater by 21.73 mg. per 100 gm. and this difference presumably represents phosphoprotein (Schmidt and Thannhauser, 1945; McCarter and Stelges, 1948) and, of course, experimental error.

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[4] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH AGE EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	0.041	0.049 ±0.002* 0.001†	0.010 ±0.0004 0.001	0.058 ±0.002 0.001	0.061 ±0.002 0.001	0.014 ±0.0006 0.001	0.527	0.108
20 day	22	0.209	0.231 ±0.006 0.001	0.055 ±0.001 0.001	0.0288 ±0.010 0.001	0.265 ±0.008 0.001	0.078 ±0.002 0.001	2.453	0.562
30 days	21	0.578	0.501 ±0.014 0.001	0.120 ±0.003 0.001	0.623 ±0.014 0.001	0.544 ±0.013 0.001	0.193 ±0.006 0.001	5.318	1.214
40 day	22	1.724	1.214 ±0.045 0.001	0.240 ±0.010 0.001	1.432 ±0.037 0.001	1.275 ±0.041 0.001	0.585 ±0.023 0.001	12.873	2.432
60 day	20	2.520	1.475 ±0.056 0.001	0.304 ±0.012 0.010	1.838 ±0.068 0.001	1.568 ±0.006 0.001	0.864 ±0.036 0.010	15.641	3.079
Normal Adult (180 day)	27	3.100	1.901 −0.042	0.387 −0.009	2.366 −0.052	2.053 −0.043	1.024 −0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

Cytological and Cytochemical Observations

Normal Adult (One hundred and eighty days). The histologic and cytologic pictures are well known and need not be detailed here. Reference to them will be made in the course of the discussion since the changes in some of the phosphorus fractions are explained best in terms of changes in the cellular population of the testis.

The Feulgen preparations showed that the nuclei of the fibroblasts, endothelial cells and mast cells which are present in the intertubular areas are rich in fine granules of DNA while those of the Leydig cells contain only a few coarse granules. The sperm heads are Feulgen-positive throughout, and the nuclei of spermatocytes and spermatogonia give a strong positive reaction. The nuclei of the spermatids are strikingly low in DNA and the same is true for the Sertoli cell nuclei.

The methyl green pyronin preparations indicate the presence of considerable basophilia in the cytoplasm of the Leydig cells and much smaller amounts in other intertubular cells. All of the tubular cells possess cytoplasmic basophilia, the amount being proportional to the amount of cytoplasm in the cells. The Sertoli cells and spermatogonia appear to contain a little more than the others, and in the area where sperm maturation is

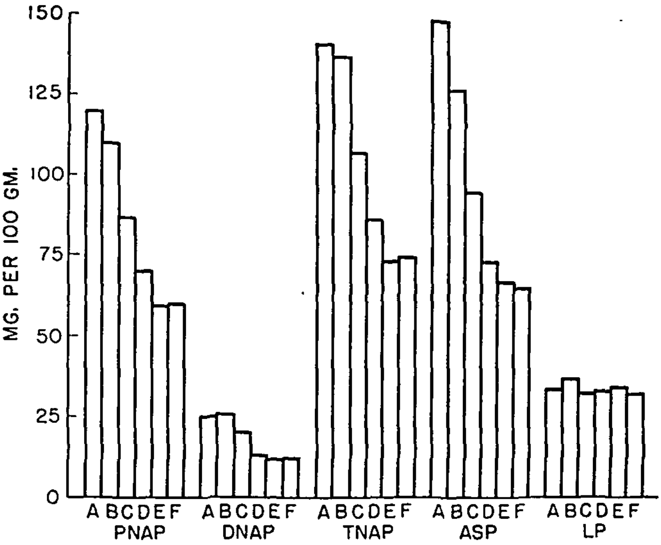

FIG. 1. Graph of the mean concentrations of the five P fractions for each age.

A = 10-day	C = 30-day	E = 60-day
B = 20-day	D = 40-day	F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

taking place there are numerous extracellular, red, basophilic droplets. These droplets are not seen in all tubules but apparently coincide with a phase in the spermatogenic wave.

Ten-Day-Old Series. The testis of a ten-day-old rat contains occasional intertubular elements, which appear to be somewhat differentiated in the direction of Leydig cells. However, most of the cells in these areas are various types of connective tissue elements. The tubules contain Sertoli cells, actively dividing spermatogonia, and possibly a few young spermatocytes.

The intertubular cell nuclei give a Feulgen reaction comparable to that seen in the cells of these areas in the adult testis. Much the same is true of the nuclei of the tubular cells; indeed, the reaction for the Sertoli cells is somewhat more marked than in the Sertoli cells of the adult testis.

Those cells in the intertubular areas which appear somewhat differentiated are high in cytoplasmic basophilia while the mesenchymal cells give a weak reaction since they contain very little cytoplasm. In all tubular cells the cytoplasm is strongly basophilic.

Twenty-Day-Old Series. There are no Leydig cells in the testis of a twenty-day-old rat, the intertubular areas being made up of connective

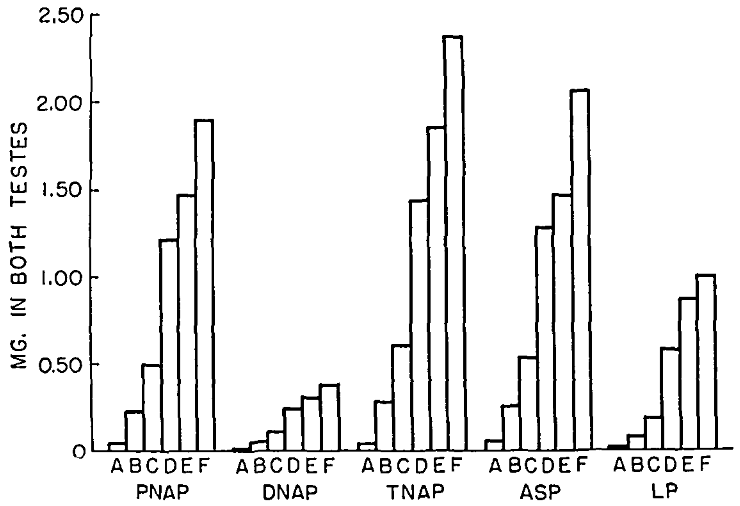

FIG. 2. Graph of the mean absolute values of the five P fractions for each age.

A = 10-day
B = 20-day
C = 30-day
D = 40-day
E = 60-day
F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

tissue with a few mast cells, capillaries and other small blood vessels. The tubules contain Sertoli cells along with spermatogonia and spermatocytes.

The nuclei of all intertubular cells give a positive Feulgen reaction of about the same intensity as in similar cells of the adult testis. The Sertoli cell nuclei show a characteristic light diffuse pattern and the spermatogonia and spermatocyte nuclei both are rich in Feulgen positive material.

As in the ten-day-old testis the mesenchymal elements have little cytoplasm and show very little basophilia. The cytoplasm of the Sertoli cells, spermatogonia and spermatocytes is more basophilic than that of like cells in the adult testis.

Thirty-Day-Old Series. The testis of a thirty-day-old rat contains a few Leydig cells and the tubules have Sertoli cells, spermatogonia, spermatocytes and some spermatids.

The nuclei of the cellular elements of both the tubular cells and the intertubular areas give a Feulgen reaction similar to that seen in the normal adult. The Leydig cells have only a small amount of Feulgen-positive material and the spermatids give a weak reaction.

In methyl green pyronin preparations the Leydig cells show cytoplasmic basophila as do the Sertoli cells, spermatogonia, spermatocytes and spermatids. The Sertoli cells and spermatogonia appear to stain a little more deeply than the spermatocytes and spermatids.

Forty-Day-Old Series. With the appearance of some sperm the forty-day-old testis assumes adult characteristics and the Feulgen picture is almost identical with that seen in normal adult testes. The amount of cytoplasmic basophilia in the testes of animals of this series appears to be in general a little greater than that present in adult testes.

Sixty-Day-Old Series. The testes of sixty-day-old rats are identical cytologically and cytochemically with those of normal adult rats.

DISCUSSION

The observations on the normal adult rat testis which have been made in this investigation confirm, in most respects, such information as is available. Chemical data for all fractions are essentially in agreement with those reported by McCarter and Stelges (1948) with the exception of the LP fraction which they found to be 59 mg. per 100 gm. of tissue as compared with our value of 32.72 mg. per 100 gm. of tissue. The discrepancy may be due in part at least to the fact that we did not use the extensive extraction procedure which they employed. However, in our experience and in that of Schneider (1946), such a laborious fractionation did not materially increase the yield of LP. It is quite possible that on the basis of a limited number of analyses such as they carried out an inconsistently high value could easily be obtained.

The cytochemical observations on the normal adult testis which have been made in this study are entirely consistent with those reported previously. Brachet (1933, 1940, 1941) has shown that in insects and amphibians the spermatogonia contain more pentose nucleic acid than the other germ cells. In our preparations we noted more cytoplasmic basophilia in the peripheral cells of the tubules; however, it was somewhat difficult to decide whether or not this greater amount was due entirely to spermatogonia or whether the Sertoli cells were also involved. Caspersson (1933, 1936) working with grasshopper testes has shown that the amount of PNA is greatly increased in the older spermatocytes. It was our experience that the spermatocytic as well as the spermatogonial nuclei give a strong Feulgen positive reaction. This is in agreement with the theses of Brachet and Caspersson that the synthesis of DNA occurs at this time and that it is dependent on the amount of PNA present in spermatogonia and primary spermatocytes. We also found that the spermatid nuclei stained very lightly with the Feulgen reagent, an observation which is easily reconciled when we consider that the spermatid nuclei contain a haploid number of chromosomes. The occurrence of large basophilic droplets in the region of sperm maturation is particularly striking in view of evidence presented by McEnery and Nelson (1950, 1951) that there is accumulation of sudanophilic and Schiff positive lipid in the same distribution. Two possible explanations may be offered for this finding. Lipoprotein complexes in the blood are well known (Troensgaard and Koudahl, 1926; Machebeouf, 1929; Machebeouf and Sandar, 1932; and Turner and Gibson, 1932) and it would seem possible that such a complex arising as a nutritive substance from the Sertoli cells, whose function is believed to be sustentacular and nutritive (Elftman, 1950; Nelson, 1951), would be visualized by lipid procedures as well as those for nucleoprotein. The second and possibly more reasonable explanation, in view of the fact that the appearance of these droplets coincides with a phase in the spermiogenic wave (Roosen-Runge, 1950, 1951), is that they arise from the sloughed cytoplasm of the spermatid during its metamorphosis. The latter explanation would be in good agreement with the observations of Claude (1939, 1940, 1941, 1949) that pentose nucleic acid exists in the cytoplasm in combination with phospholipid. Thus it is possible that the remnants of such a pentose nucleic acid-phospholipid complex are released when the spermatid is transformed into a spermatozoon and are visualized by the respective cytochemical procedures.

During the attainment of the adult condition changes in the different phosphorus fractions follow rather uniform patterns and the cytochemical observations are in good agreement with the patterns for the concentration of the nucleic acids. The concentrations of all fractions, with the exception of LP which remains relatively constant, decrease progressively as the age of the testis increases (Fig. 1), while the absolute values (amounts in both testes) for all of the phosphorus fractions increase progressively with the age of the testis (Fig. 2).

Adequate interpretation of these findings requires that we examine the morphologic changes which take place in the testis between ten and one hundred eighty days of age. In the first place there is approximately an eighty fold increase in weight. This is brought about by increases in the size of cells and by increases in their number. Secondly, the attainment of maturity involves the appearance of new cell types. The former, as we shall point out, appears to exert the major influence on the absolute values of the phosphorus fractions while the latter exerts more influence on the variations in concentration.

Two points must be developed in order to clarify this. In the first place it is well known that PNA is found chiefly in the cytoplasm. This is apparently also the case for the ASP fraction. Therefore tissue made up of cells having a large amount of cytoplasm (low nuclear-cytoplasmic ratio) will be high in those two substances whereas tissue made up of cells having little cytoplasm (high nuclear-cytoplasmic ratio) will have low concentrations of these substances. DNA on the other hand is for the most part (probably exclusively) in the nucleus and is actually a very important constituent of the chromosomes. Thus the DNA content is affected by the nuclear cytoplasmic ratio of cells making up a given tissue. Furthermore, in the case of the testis there are cells with haploid as well as cells with diploid numbers of chromosomes. This will also influence the content of DNA. In fact, Boivin, Vendrely and Vendrely (1948) found that the nuclei of somatic cells from animals of a given species have twice as much DNA as the corresponding sperm (haploid) nuclei. Thus in the adult testis the concentrations of the various phosphorus fractions will be a function of the numbers of the various cell types present at that time.

In the second place attention must be directed to the cytologic picture of the testis at each age included in this study. At ten and twenty days of age the seminiferous tubules of the rat testis contain only cells which have considerable cytoplasm, namely Sertoli cells, spermatogonia and primary spermatocytes, while at thirty days secondary spermatocytes and spermatids have made their appearance and at forty days sperm are in evidence. After forty days no new cell types are added and further changes involve only increases in the numbers of cells, particularly those of the more mature types. Three significant generalizations can therefore be made with regard to the growth and maturation of the testis. First, as new cell types are added the total nuclear-cytoplasmic ratio of the cells of the testis changes from a lower to a higher value. Secondly, the last three cell types to appear, namely, the secondary spermatocytes, spermatids and sperm, reduce the total number of chromosomes per cell in the testis. Thirdly, the great increase in numbers of these latter cell types influence not only the concentrations by dilution of the cell population but also the absolute values by adding materials to the testis as a whole.

That the PNAP concentration was highest at ten and twenty days is entirely consistent with the cytologic picture. When, however, the cytoplasmic basophilia was examined in these two groups, it was found to be more intense than in corresponding cells in the adult group and further, that it was somewhat greater in the ten than in the twenty day testis. Thus it would seem that testes of young rats have a higher concentration of PNA than can be accounted for entirely on the basis of the cell types which are present. Since slightly elevated values for PNA have been found generally in young growing tissues and greatly increased amounts, as compared to adult tissues, have been observed in embryonic tissues (Davidson, 1950) it is not surprising to find that the PNA values in the testes of young rats, which are similar in some respects to embryonic tissues, are markedly higher than in adult testes. It is worth noting that from the tenth to the twentieth day the testis undergoes a fivefold increase in weight and from the twentieth to the thirtieth day a threefold increase. Therefore, the high concentration can be attributed in part at least to the accelerated rate of growth in young testes.

At thirty and forty days the lower PNA concentrations are entirely

ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT UNDER EXPERIMENTAL CONDITIONS WHICH IMPAIR SPERMATOGENESIS[1,2]

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IN SPITE of the concerted efforts which have been made to clarify the functional position and metabolic significance of nucleic acids very little in the way of established interpretations can be offered. Caspersson (1950) and Brachet (1950) have discussed the significance of nucleic acids in the synthesis of cellular proteins, Claude (1940, 1941, 1942, and 1948) has shown that cytoplasmic pentose nucleic acid (PNA) occurs in combination with phospholipids and Hevesy (1948) and Davidson (1950) have pointed out certain relationships between PNA and the components of the acid-soluble phosphorus (ASP) fraction.

It has been reported that fasting and protein-poor diets (Davidson and Waymouth, 1944; Davidson, 1945), ischemia (Drochmans, 1947), adrenocorticotrophic hormone and high carbohydrate diet (Baker, *et al.*, 1948), and hypophysectomy (Geschwind, Li and Evans, 1950) all cause a decrease in pentose nucleic acid (PNA) but no change in desoxypentose nucleic acid (DNA) in the liver. McShan *et al.* (1950) reported a marked increase in PNA but only a slight increase in DNA in the crop glands of pigeons treated with lactogenic hormone. An increase in both PNA and DNA has been observed in the livers of pregnant rats (Campbell and Kosterlitz, 1947) and Stutz and Verzar (1947) reported that thymectomy did not alter the nucleic acid content of the rat liver. Mitchell (1942, 1943) has observed that x-irradiation of various normal and cancerous human tissues produces an accumulation of PNA in the cytoplasm, and Stowell

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[2] This paper is a part of the content of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy of the Graduate College of the State University of Iowa, February 1952.

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(1945) reported that x-irradiation prevents the synthesis of DNA in cancerous tissue. The studies of Errera (1947) indicate a direct effect of x-irradiation on the chemical properties involved in the rigidity of the nuceloprotein complex *in situ.*

In a previous paper (McEnery and Nelson, 1952) we pointed out the scarcity of chemical and cytochemical observations on nucleic acids and other phosphorus containing constituents in the testis and reported such information for the testes of rats ranging in age from ten to one hundred and eighty days.

This report offers similar observations on the testes of hypophysectomized, estrogen-treated, x-irradiated and cyptorchid rats. The experimental procedures to which these rats were subjected were chosen for their well-known effects on spermatogenesis.

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory and of the Sprague-Dawley strain obtained from a commercial breeder were used in this study.

Animals were hypophysectomized[4] at weights varying from 150 to 250 gm. and were sacrificed 30 days post-operatively. All animals were examined for completeness of operation.

In the estrogen-treated animals treatment was initiated when the animals weighed approximately 150 gm. Subcutaneous injections of alpha estradiol,[5] were made in 50 and 100 gamma doses daily for 30 to 45 days.

In the x-ray series animals weighing 200 to 250 gm. were given 600 roentgen units applied in a single dose to the caudal half of the body and were autopsied 30 days later.[6]

In the cryptorchid series animals weighing 200–250 gm. at the time of operation were made cryptorchid by anchoring the testes to the anterior abdominal wall. One group was sacrificed 80 days post-operatively and the other 200 days after operation.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). Duplicate determinations were made on testicular tissue from each animal. All were wet weight determinations and moisture contents were checked in each group.

Cytological and Cytochemical Preparations

Portions of the testes were prepared for cytological and cytochemical observation according to the methods described in the first paper of this series (McEnery and Nelson, 1952).

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[4] The hypophysectomized rats were obtained from the Hormone Assay Laboratory of Chicago, Illinois.

[5] We are indebted to Dr. Edward Henderson of the Schering Corporation for the alpha estradiol.

[6] We are indebted to the staff of the Radiation Research Laboratory of the State University of Iowa for invaluable assistance in irradiating these animals.

TABLE 1. THE MEAN CONCENTRATIONS OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN CONCENTRATIONS OF PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. PER 100 GM. OF FRESH TISSUE

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	87.14 ±1.664* 0.001†	23.45 ±0.481 0.001	109.87 ±1.865 0.001	81.73 ±1.857 0.001	34.47 ±1.010 0.20	923.68	236.84
Estrogen	21	86.01 ±1.993 0.001	23.24 ±0.576 0.001	110.53 ±1.653 0.001	95.28 ±1.230 0.001	33.44 ±0.512 0.20	911.70	234.72
X-ray	19	67.66 ±1.021 0.001	14.63 ±1.516 0.050	82.25 ±1.646 0.001	81.26 ±2.186 0.001	39.60 ±0.989 0.001	717.19	147.76
Cryptorchid (80-day)	18	66.52 ±2.054 0.010	17.32 ±2.235 0.010	84.04 ±2.015 0.001	78.37 ±2.349 0.001	54.85 ±1.747 0.001	705.11	174.93
Cryptorchid (200-day)	24	67.24 ±1.596 0.001	19.42 ±1.676 0.001	85.75 ±1.300 0.001	65.90 ±1.889 0.20	56.55 ±0.962 0.001	712.74	196.14
Normal Adult (180-day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.29	124.23

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentration of phosphorus in the PNA, DNA, TNA, AS and L fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the differences between the means of the values obtained for these fractions in the animals of the various experimental groups as compared with the equivalent values in normal adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2, and the values for concentration and absolute amounts are represented graphically in Figures 1 and 2. It will be noted that the concentrations of the various phosphorus fractions showed varying degrees of increase in the different experimental series as compared to the corresponding values for the normal adult rat. On the other hand the absolute values for all fractions were consistently lower than those observed in the normal adult.[7]

The water content of the testes in the experimental groups did not differ more than five per cent from that observed in testes of normal adult rats.

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[7] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	0.625	0.548 +0.020* 0.001†	0.146 ±0.004 0.001	0.690 ±0.024 0.001	0.509 ±0.014 0.001	0.216 ±0.009 0.001	5.809	1.481
Estrogen	21	0.766	0.657 ±0.058 0.001	0.173 ±0.011 0.001	0.835 ±0.063 0.001	0.731 ±0.063 0.001	0.255 ±0.022 0.001	6.965	1.755
X-ray	19	0.694	0.469 ±0.012 0.001	0.101 ±0.010 0.001	0.570 ±0.015 0.001	0.564 ±0.019 0.001	0.276 ±0.012 0.001	4.975	1.025
Cryptorchid (80-day)	18	0.755	0.503 ±0.020 0.001	0.130 ±0.005 0.001	0.635 ±0.022 0.001	0.601 ±0.035 0.001	0.416 ±0.019 0.001	5.331	1.318
Cryptorchid (200-day)	24	0.790	0.531 ±0.016 0.001	0.154 ±0.006 0.001	0.678 ±0.019 0.001	0.523 ±0.021 0.001	0.449 ±0.016 0.001	5.632	1.556
Normal Adult (180-day)	27	3.100	1.901 ±0.042	0.387 ±0.009	2.366 ±0.052	2.053 ±0.043	1.024 ±0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

Consequently differences seen in the phosphorus fractions were not influenced to any appreciable degree by changes in the moisture content.

Cytological and Cytochemical Observations

The picture in the normal adult testis has been described in a previous paper (McEnery and Nelson 1952).

Hypophysectomized Series. In the testis of the hypophysectomized rat ordinary histologic preparations show that the Leydig cells are atrophic and the tubules contain only Sertoli cells, spermatogonia and spermatocytes.

All of the nuclei of the intertubular elements including the Leydig cells, show a strong Feulgen reaction while the tubules (except for some sloughed spermatocytes which stain deeply) present a picture similar to that seen in the same cells of the normal testis.

While the intertubular cells contain little cytoplasm that which is present is strongly basophilic in methyl green pyronin preparations. The degree of cytoplasmic basophilia in the tubular cells, with the exception of some degenerating spermatocytes which are strongly basophilic, is com-

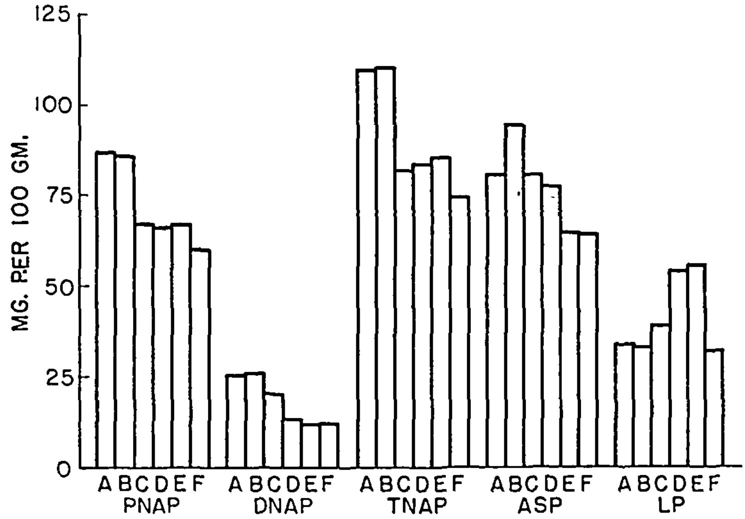

FIG. 1. Graph of the mean concentrations of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy C = X-ray E = Cryptorchid (20-day)
B = Estrogen-treated D = Cryptorchid (80-day) F = Normal Adult
PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

parable to that observed in the same cells of normal testes. In some of the degenerating cells even the nucleus is stained red and occasional red droplets occur free in the tubular lumens.

Estrogen Series. The testes of estrogen treated animals show atrophic Leydig cells and the tubules contain only Sertoli cells, spermatogonia, and spermatocytes. This picture as seen in ordinary histologic preparations is essentially the same as in hypophysectomized animals and is due to the lack of gonadotrophic hormone.

The Feulgen reaction is intense in the nuclei of all intertubular cells. The nuclei of the Sertoli cells contain little Feulgen-positive material while those of the spermatogonia and spermatocytes stain deeply.

The quantity and distribution of cytoplasmic basophilia throughout the testes of these animals appears to be essentially the same as has been described for the testes of hypophysectomized animals. There are, however, few of the degenerating spermatocytes which are so highly basophilic in the latter animals.

X-ray Series. The x-irradiated testes show intertubular areas with an apparent increase in both number and size of the Leydig cells. In x-irradiated testes an extremely variable picture is found in the tubules, some containing only Sertoli cells while others contain sperm, occasional spermatids, a few large basal cells, and some unidentifiable cellular remnants.

The Feulgen reaction in the cell nuclei of the intertubular areas is essentially similar to that seen in the cells of these areas in normal adult testes. Within the tubules the Sertoli cells appear to contain a normal amount of Feulgen-positive material and such other cells as are present give an essentially normal reaction except for the nuclei of the basal cells and cell remnants, both of which stain deeply.

In the methyl green pyronin preparations the cytoplasm of the intertubular cells show approximately the same amount of basophilia as that of corresponding cells in the normal adult testis (a few Leydig and mast cells appear to show more). The residual tubular cells are a little more basophilic than cells of the same types in normal adult testes.

Cryptorchid Series. The testes in both of these groups are essentially similar and will be discussed together. They show the customary apparent

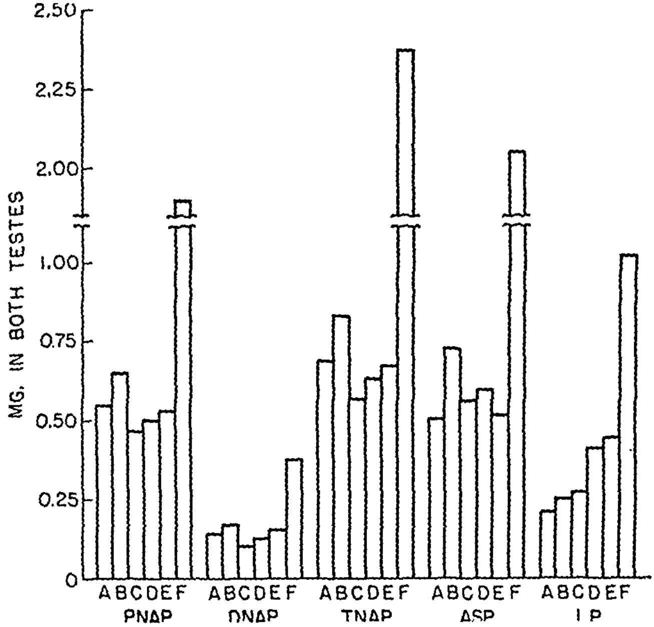

FIG. 2. Graph of the mean absolute values of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy C = X-ray E = Cryptorchid (200-day)
B = Estrogen-treated D = Cryptorchid (80-day) F = Normal Adult
PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

increase in the number and size of the Leydig cells. The tubules vary with respect to the types of cells present, some having Sertoli cells only and others, in addition, spermatogonia and even occasional spermatocytes.

With the Feulgen stain all of the nuclei of the tubular and intertubular cells show essentially the same reaction as is seen in the corresponding cells of normal adult testes.

The methyl green pyronin preparations reveal an increased amount of cytoplasmic basophilia in the Leydig cells while other intertubular cells show normal quantities. The degree of cytoplasmic basophilia in the tubular cells is also essentially normal.

DISCUSSION

In the first paper of this series (McEnery and Nelson, 1952) the significance of observations on the normal adult testis has been discussed and we have pointed out that variations in the concentration of the phosphorus fractions at various ages was chiefly due to the types of cells which occur in the seminiferous tubules at those ages. The reader is referred to that paper for a detailed discussion of the constancy of position of PNA and DNA in the cell, the relationship of those substances to the amount of cytoplasm and the presence of nuclei with diploid or haploid numbers of chromosomes, and the apparent relationship between the PNAP and ASP fractions. In the LP fractions, on the other hand, such relationships are not evident.

The PNAP concentrations in testes from hypophysectomized and estrogentrated animals showed substantial increases over those found in testes of normal adult rats. Physiologically these two conditions are similar, in that gonadotrophins are absent, and the testicular tubules contain only Sertoli cells, spermatogonia and primary spermatocytes. Furthermore, cytoplasmic basophilia in these cells is no greater than that seen in the normal adult (except for a few disintegrating cells which could not alter the picture significantly). Thus it seems likely that the increased PNAP concentration in these two groups is probably due to the presence of cells with relatively large amounts of cytoplasm rather than to any specific effect of the treatment on PNAP since the cells listed above contain larger amounts of cytoplasm than do secondary spermatocytes, spermatids and spermatozoa.

The testes of x-irradiated animals showed some increase in the concentration of PNAP. These results agree with those of Mitchell (1942, 1943) in spite of the fact that our animals were sacrificed thirty days following treatment while Mitchell examined his tissue within nine hours post-irradiation. The possibility exists that in our study we were dealing with some measure of spermatogenic repair although histologic evidence of such repair was not evident. A clear cut evaulation of the testicular picture in terms of the content of nucleic acids is somewhat difficult in this series owing to the variable cellular content of the tubules. It can be stated, however, that the Sertoli cells were a constant feature of the tubules, that some of the tubules contained flattened basal cells and that both of these cells were significantly basophilic. Since the spermatoids and sperm contain little cytoplasm it would appear that the Sertoli cells and basal cells account for the majority of the PNA.

The increases in PNA concentration in the cryptorchid groups are not excessively large. Since the cell types which are present in the tubules contain considerable cytoplasm and since the Leydig cells show increased basophilia one might have expected higher values. Apparently the Sertoli cells in these testes are not sufficiently rich in PNA to provide the substantial increases seen in some of the other experimental series and the Leydig cells, since they are closely grouped and relatively more numerous may give the illusion of increased basophilia.

The concentrations of DNAP in the testes from hypophysectomized and estrogen-treated animals were double those found in normal adult animals. This is easily understood when we note that only cells whose nuclei possess diploid numbers of chromosomes were present.

Considerable variation was seen in the concentrations of DNAP in the testes of x-irradiated animals. However, if we take into account the variations which were encountered in the histologic condition of these testes the values for concentration of DNAP are seen to be consistent with the histologic picture. To assess this relationship more accurately it would be necessary to study a large series of animals in which individual analyses would be correlated with the varied histologic patterns in the same testes.

In the two groups of cryptorchid animals the values for the concentrations of DNAP present a somewhat baffling problem. All of the cells contain diploid numbers of chromosomes. Thus one would expect the concentrations of DNAP to be double the normal adult value, as was the case for hypophysectomized and estrogen-treated rats. Actually the concentrations were between the values observed for hypophysectomized and estrogen-treated groups and for normal adult animals. Since the Feulgen reaction in the testes of hypophysectomized and estrogen-treated animals is similar to that seen in normal adult testes it is possible that the Sertoli cells, which appear to be the predominant type of cell in the testes of cryptorchid rats, do not have as high a content of DNA as do the spermatogonia and spermatocytes which are abundant in the testes of hypophysectomized and estrogen-treated rats. That these latter cells contain larger amounts of DNA than are present in other intratubular cells has been shown by Caspersson's (1933, 1936) studies utilizing absorption microspectrophotometric techniques.

The concentration of ASP in the testes of hypophysectomized animals paralleled that of PNAP both being higher than the corresponding values for normal adult testes and, as noted earlier for PNAP, are probably related to the types of cells which are present. On the other hand an even higher value for ASP was seen in the estrogen-treated group. A suggestion of an effect of estrogen upon constituents of the acid soluble phosphorus fraction may be found in the observations of Dirscherl and Hauptmann (1950) who reported that estrogens stimulate anaerobic glycolysis and respiration in liver slices and of McShan, Meyer and Erway (1947) and Case and Dickens (1948) who found that some estrogens inhibit the succinoxidase and cytochrome oxidase systems.

In the x-irradiated animals the ASP concentration closely paralleled the values for PNAP and therefore the comments made for PNAP apply in this case.

The concentration of ASP in the testes of eighty-day cryptorchid rats was somewhat higher than in normal adults while in the two hundred-day cryptorchids the concentration was similar to the normal value. We are unable to offer an explanation for these variations.

The observation that the concentration of the LP fraction in the testes of hypophysectomized and estrogen-treated rats does not differ from the normal value would seem to confirm the reports of Sinclair (1940, 1941) that the testicular phospholipids are inert.

The testes of x-irradiated animals and of the two groups of cryptorchid animals showed concentrations of LP which were significantly higher than the corresponding value for normal adult testes. It is noteworthy that the Sertoli cells are a constant feature as well as the predominant type of cell in these groups and it might be offered as a possible explanation that the Sertoli cell contains a larger quantity of phospholipid. On the other hand the experimental conditions imposed in the cryptorchid and x-irradiated animals may possibly have altered directly the phospholipid content. It is obvious, however, that the present status of our knowledge regarding phospholipid metabolism renders extremely difficult the proposal of any sound interpretation of these observations.

The absolute values for all fractions in the experimental series were lower than the normal adult values. This is not surprising since the testes of all of the experimental animals were much smaller than normal and had various types of cells missing from their seminiferous tubules.

SUMMARY AND CONCLUSIONS

The testes of hypophysectomized, estrogen-treated, x-irradiated and cryptorchid rats were analyzed for pentose nucleic acid phosphorus, desoxypentose nucleic acid phosphorus, total nucleic acid phosphorus, acid soluble phosphorus and lipid phosphorus. Observations also were made on routine histologic preparations and on cytochemical preparations with the Feulgen reaction and for cytoplasmic basophilia. The latter two procedures are considered to be related to desoxypentose nucleic acid and pentose nucleic acid, respectively.

In testes from hypophysectomized and estrogen-treated animals the concentrations of all phosphorus fractions, except the lipid fraction, showed definite increases over the normal adult values.

In testes from x-irradiated animals moderate increases in the concentrations of all phosphorus fractions were seen.

In the cryptorchid rats a striking increase in the concentrations of the lipid fraction and a definite increase in the concentration of desoxypentose nucleic acid phosphorus were observed. The other fractions in testes from these animals showed only moderate increases in concentration.

The absolute values for all fractions in each of the experimental series were considerably lower than the equivalent values for normal adult animals. This is in keeping with the reduced size of the testes.

The concentrations of the various phosphorus fractions in the testes of hypophysectomized and estrogen-treated animals showed rather definite patterns which appeared to depend upon the types of cells which are present in such testes. On the other hand the values for the concentration of the several phosphorus fractions in testes of x-irradiated and cryptorchid animals present certain inconsistencies which may be due to physical and/or chemical alterations which are superimposed on such changes as may have occurred as a result of alterations in the cell populations. Further evaluation of these factors and their influences will require additional and more detailed studies.

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NOTES AND COMMENTS

SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE SHEEP AND COW

Although serum ADS has been found in rats (Birnie *et al.*, 1949); in humans (Lloyd and Lobotsky, 1950; Hawker, 1952a and 1952b); and in the kangaroo rat (Ames and van Dyke, 1952) a quantitative estimation of it in the sera of sheep and cows has not previously been reported.

This note reports the finding of serum ADS in sheep and cattle.

PROCEDURE

The method of Birnie *et al.* (1949)for measuring ADS in rats' sera was used in these experiments; the ADS units have been quantitated in terms of milliunits of pitressin *(Parke Davis pitressin 20 u. per ml.). Serum ADS was assayed in the following groups:*

A. *Sheep*
  (1) Non-pregnant, non-lactating ewes (8 animals)
  (2) Pregnant, non-lactating ewes (4 animals)
B. *Cattle* (Dairy and Beef)
  (1) Non-pregnant, non-lactating dairy cows (8 animals)
  (2) Non-pregnant, lactating dairy cows (8 animals)
  (3) Pregnant, non-lactating dairy cows (8 animals)
  (4) Pregnant, lactating dairy cows (6 animals)
  (5) Dairy bull (1 animal)
  (6) Non-pregnant, non-lactating beef cows (3 animals)
  (7) Beef bull (1 animal)

The dairy cows were graded into Grades I, II and III, according to their capacity to produce milk. The Grade I cows belonged to a record-holding herd for milk production in Queensland, Australia.

Table 1. ADS concentration in sheep during non-pregnancy and pregnancy

Species	Type	No. assayed	No. of assays	mμ ADH/ml. serum	mμ ADH/ml. (mean)
Sheep	non-pregnant & non-lactating	8	8	0.00 0.50 0.35 0.75 0.30 2.00 2.10 1.25	1.16
Sheep	pregnant & non-lactating	4	4	4.60 5.85 6.10 3.85	5.10

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RESULTS

A. Serum ADS in Sheep

Serum ADS was found in both the non-pregnant and pregnant sheep, with a higher concentration occurring during pregnancy, as shown in Table 1.

B. Serum ADS in Cattle

An antidiuretic substance was also determined in the sera of the dairy and beef cattle examined, and the results are presented in Table 2.

TABLE 2. ADS CONCENTRATION IN DAIRY AND BEEF CATTLE DURING NON-PREGNANCY, PREGNANCY AND LACTATION

Cattle	Type	No. assayed	Grade	mμ ADH/ml. serum	Mean	Reproductive state
Dairy	non-pregnant & non-lactating	2	1	4.00 3.20	3.60	nulliparous pre-puberal
	non-pregnant & non-lactating	3	1	5.40 5.65 5.05	5.37	nilliparous post-puberal
	non-pregnant & lactating	4	1	7.70 5.80 7.10 8.00	7.15	parous
	pregnant & non-lactating	4	1	5.90 7.15 7.50 7.55	7.02	—
	non-pregnant & non-lactating	4	2	6.00 5.05 7.35 7.70	6.52	parous
	non-pregnant & lactating	4	3	4.95 3.85 5.80 3.30	4.47	parous
	pregnant & non-lactating	4	3	6.20 4.15 5.00 5.15	5.12	—
	pregnant & lactating	6	1	7.25 4.35 3.20 5.95 5.75 5.30	5.30	—
	Bull	1	1		3.45	—
Beef	non-pregnant & non-lactating	2		4.30 3.40	3.85	nulliparous post-puberal
	Bull	1			2.90	—

Reference to Table 2 shows that in any type examined the better the dairy cow is graded for milk production, the higher is her serum ADS level.

SUMMARY AND CONCLUSIONS

(1) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn sera of sheep and cattle.

(2) In ewes the serum ADS level is higher during pregnancy than in the non-pregnant state.

(3) In dairy cows of Grade I milking strain the serum ADS level is lowest in prepuberal heifers, and shows increments with sexual maturity, pregnancy and lactation.

Acknowledgements

I am grateful to the National Health and Medical Research Council (Australia) for their grant which enabled this work to be undertaken.

I also wish to thank Professor T. K. Ewer, The Veterinary School, University of Queensland, for the use of his laboratory.

My gratitude is also extended to Mr. A. McDowall of the same address for his valuable assistance in this experiment, and to Miss Joan Hunter, Department of Physiology, University of Queensland, for technical help.

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SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE HUMAN FEMALE

A substance possessing antidiuretic properties (and henceforth referred to here as ADS) was found in the serum of rats by Birnie *et al.* (1949); in the human by Lloyd and Lobotsky (1950), and by Hawker (1952a and 1952b); and in the kangaroo rat, Dipodomys merriami, by Ames and van Dyke (1952). Birnie *et al.* (1949) consider that ADS found in rat serum is probably identical with the antidiuretic hormone (ADH) produced by the posterior pituitary gland.

This paper reports the finding of ADS in the adult human female during the normal menstrual cycle, pregnancy and the immediate post partum period.

PROCEDURE

Serum ADS was assayed according to the method of Birnie *et al.*, (1949). A graph was constructed correlating ADS units with milliunit concentrations of freshly prepared

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Received for publication June 17, 1952.

pitressin (Parke Davis. 20 u. per ml.). Serum ADS was assayed in the following groups:

(1) Healthy non-pregnant females—mostly (medical) students—aged between 17–22 years; in all, 65 assays were done on 28 individuals at different times during the menstrual cycle.

(2) Healthy pregnant women—usually from the 10th or 12th week following the last menstrual period; a total of 61 assays were performed on 24 women.

(3) Healthy women in immediate post partum period; 50 assays were done on 11 women in the first 4–5 days after delivery.

RESULTS

This work shows that ADS is present in the serum of the healthy, cyclically menstruating female. Figure 1 shows clearly that during the menstrual cycle the serum ADS level is most commonly 0–1 mμ ADH/ml. of serum; the arithmetic mean of the 65 assays examined was 1.43 (S.D. ±1.18) mμ ADH/ml. of serum. Figure 1 also reveals that during pregnancy the serum ADS level is higher than during the menstrual cycle, and the distribution is wider. Here the serum ADS level is most commonly 5–6 mμ ADH/ml. of serum; the mean of the 61 assays was 4.53 mμ (S.D. ±1.34 ADH/ml. of serum.

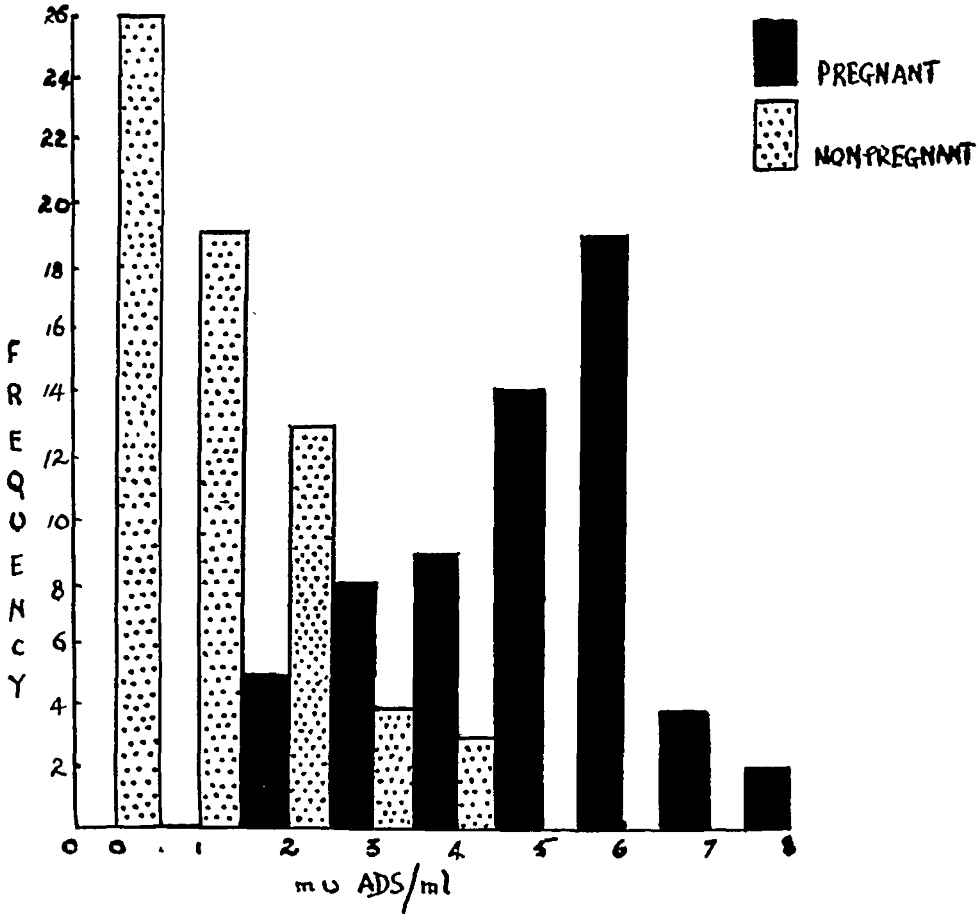

FIG. I. Histogram of serum ADS levels in pregnant and non-pregnant women.

The serum ADS levels in the weeks of the menstrual cycle are expressed in Table 1.

TABLE 1. ADS CONCENTRATION DURING THE MENSTRUAL CYCLE

		Milliunits of ADH/ml. serum	
Menstrual Cycle	Whole Cycle	1.43 (S.D. ±1.18)	Mean of 65 assays
	0– 7 days	1.48	Mean of 17 assays
	8–14 days	0.95	Mean of 10 assays
	15–21 days	1.72	Mean of 12 assays
	22–28 days	1.45	Mean of 15 assays

Some results are not included, as a record of the time in the menstrual cycle, when the assays were done, was not obtained.

The serum ADS levels during pregnancy are presented in Table 2, from which it will be seen that the level appears to remain comparatively steady throughout pregnancy with perhaps a slight over-all upward trend as pregnancy advances.

TABLE 2. ADS CONCENTRATION DURING NORMAL PREGNANCY AND FIRST DAY POST PARTUM

Time	Milliunits ADH/ml. serum (mean)	
1st Trimester	3.69	(4 assays)
2nd Trimester	4.21	(15 assays)
3rd Trimester	4.37	(21 assays)
Last month of pregnancy	4.37	(9 assays)
1st day post partum	5.50	(11 assays)

Some results are not included in the table because a record of the period of gestation was not obtained.

Immediately after parturition the serum ADS levels are higher than are found in non-pregnant women, and reference to Table 3 will show that a correlation appears to exist between the level of serum ADS and the onset of lactation. Here there occurred an initial post partum fall in the serum ADS level, and lactation began within a few hours after the serum ADS approached or reached its lowest recorded initial level. In no instance did lactation begin before the *initial* fall in the serum ADS level.

TABLE 3. ADS CONCENTRATION AND ONSET OF LACTATION

Case	Days after parturition				
	Milliunits ADH/ml. serum				
	1	2	3	4	5
1	7.0	5.85	5.0	*4.15*	5.2
2	4.9	4.45	*4.0*		
3	3.45	4.85	*2.35*	6.15	
4	*2.5*	3.15	3.35	5.1	
5	5.95	5.2	*2.9*	3.0	5.1
6	4.45	4.3	*3.35*	7.1	2.3
7	4.4	3.84	*0*	7.2	3.9
8	7.85	*3.3*	5.3		
9	5.5	*5.15*	7.2	2.95	4.1
10	5.6	5.6	5.85	*3.45*	3.9
11	5.9	*3.1*	4.9	2.75	5.45

Italicized figures = Lactation commenced within hours of this point.

The mean concentrations of ADH/ml. of serum for the eleven cases on the first day post partum and at the onset of lactation were 5.50 (S.D. ±1.29) and 3.11 (S.D. ±1.39) respectively. (First post partum reading for Case No. 4 excluded as lactation commenced on the first day post partum.)

SUMMARY AND CONCLUSIONS

(i) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn serum of women during the normal menstrual cycle, normal pregnancy and early post partum period.

(ii) The serum ADS level is higher during pregnancy than during the menstrual cycle.

(iii) It is postulated that this high level during pregnancy accounts for some of the water retention of pregnancy.

(iv) A correlation appears to exist between the level of serum ADS after parturition and the onset of lactation. In each of the eleven cases examined, lactation always began after an initial fall in the serum ADS level.

Acknowledgements

I am grateful to the National Health and Medical Research Council (Australia) for their grant which enabled this work to be undertaken.

I wish to thank Professor W. V. Macfarlane of the Department of Physiology and Professor, H. J. G. Hines of the Department of Biochemistry, University of Queensland, *for their many helpful suggestions and criticisms during the course of this study.*

I am also indebted to Dr. R. Salter and Dr. K. G. Cockburn of the Brisbane Women's Hospital for their co-operation and I am pleased to acknowledge the technical assistance given to me during the course of these studies by Miss Joan Hunter, Department of Physiology, University of Queensland.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

ENDOCRINOLOGY

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THE EFFECT OF ANDROGENIC HORMONES UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE INJECTIONS AND UPON THE ANTI-INFLAMMATORY ACTION OF CORTISONE[1]

CHARLES A. WINTER, HELEN L. HOLLINGS AND ROBERT B. STEBBINS

From the Merck Institute for Therapeutic Research, Rahway, New Jersey

ZIZINE, Simpson and Evans (1950) reported that testosterone brought about a significant degree of adrenal repair in hypophysectomized animals. Since cortisone produces adrenal atrophy which resembles in many respects that seen after hypophysectomy, it was of interest to determine whether androgenic hormones would antagonize this effect of cortisone.

MATERIAL AND METHODS

Young adult male Holtzman rats were used in this study, except for the experiments where hypophysectomized rats were employed; the latter were purchased from the Hormone Assay Laboratories, Inc., Chicago. Steroids were injected in saline suspension subcutaneously, once daily. Some experiments were performed with testosterone propionate, but the androgen employed in most of the study was 17(α)-methyl-Δ^5-androstene-3(β)-17(β)-diol (methyl-androstenediol or mestenediol)* (M.A.D.). The cytochemical methods used in this investigation have been described elsewhere (Stebbins, 1951a).

RESULTS

Effect of testosterone in cortisone-treated rats. Table 1 gives the results of experiments in which cortisone was injected daily for a period of 9 days. Although adrenal atroply is not maximal within that period of time, it

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[1] Part of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Omaha, Nebraska, October 15–17, 1951, and an abstract appears in the Proceedings of that meeting (*J. Pharmacol. Exper. Therap.* **103**: 366. 1951).

* The authors gratefully acknowledge generous supplies of methylandrostenediol through the courtesy of Dr. K. W. Thompson, Organon, Inc., Orange, N. J.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 9 DAYS, MEAN VALUES FOR GROUPS OF 8 RATS

Treatment	Body weight		Thymus, mg.	Adrenals	
	Initial, g.	Final, g.		mg.	Mg./100 g. body wt.
Cortisone acetate 1.5 mg.	107	114	65	14.5	12.8
Cortisone acetate 3 mg.	98	104	24	14.1	13.5
Cortisone acetate 1.5 mg.+testosterone propionate 2.5 mg.	101	119	26	19.2	16.3
Cortisone acetate 3 mg.+testosterone propionate 2.5 mg.	109	108	18	19.0	17.8
Untreated controls	109	165	522	29.9	18.1

has been shown that most of the atrophy produced by cortisone occurs within the first 10 days (Winter, Silber and Stoerk, 1950). In the present experiment, there was not much difference in the degree of adrenal atrophy produced in 9 days by 1.5 mg. and by 3 mg. of cortisone acetate. Testosterone propionate, when given in conjunction with cortisone, prevented the adrenal atrophy to a considerable extent. It did not, however, prevent the suppression of body growth or atrophy of the thymus which is characteristic of cortisone-treated rats.

Effect of methylandrostenediol in cortisone-treated rats. When M.A.D. was given in graded doses in combination with 2.5 mgm. daily of cortisone acetate, a degree of prevention of adrenal atrophy was obtained which was directly related to the dose of M.A.D. employed up to 0.5 mgm. daily (Table 2). 0.05 mg. of M.A.D. produced no noticeable effect, but when 0.5 mg. was given to the cortisone-treated rats, the adrenals remained with-

TABLE 2. EFFECT OF GRADED DOSES OF METHYLANDROSTENEDIOL (M.A.D.) UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 19 DAYS (EXPERIMENT 1) OR 18 DAYS (EXPERIMENT 2); MEAN VALUES FOR GROUPS OF 9 TO 10 RATS

Treatment	Experiment No.	Body weight		Thymus, mg.	Adrenals	
		Initial, g.	Final, g.		Mg.	mg./100 g. body wt.
Cortisone acetate 2.5 mg.	1	106	92	23	13.0	14.3 ±0.66*
Cortisone acetate 2.5 mg.	2	120	111	23	12.9	11.8 ±0.59
Cortisone acetate 2.5 mg. +M.A.D. 0.05 mg.	1	113	106	22	14.7	13.9 ±0.60
Cortisone acetate 2.5 mg. +M.A.D. 0.1 mg.	1	106	99	20	15.0	15.1 ±0.68
Cortisone acetate 2.5 mg. +M.A.D. 0.25 mg.	1	111	98	20	15.8	16.2 ±0.89
Cortisone acetate 2.5 mg. +M.A.D. 0.5 mg.	2	115	116	25	23.7	20.5 ±1.45
Methylandrostenediol 0.5 mg.	2	125	188	381	43.8	24.2 ±1.83
Uninjected controls	1	111	190	435	39.3	21.3 ±1.81
Uninjected controls	2	125	198	440	42.7	21.8 ±1.07

* Standard error.

in the limits of normal size for rats of corresponding body weights. Higher doses of M.A.D. (not shown in the table) produced no greater effect upon adrenal weight. Thymus atrophy was virtually complete in all the animals treated with cortisone regardless of whether or not M.A.D. was also administered. The M.A.D. did not counteract the suppression of body growth by cortisone. M.A.D. by itself, in a dose of 0.5 mg. daily for 18 days produced about a 13 per cent decrease in thymus weight, but body weight and adrenal size were not significantly affected.

Histological examination of the adrenal glands of these animals demonstrated that the changes produced by cortisone could be largely prevented by concurrent administration of M.A.D. Cortisone produced a decrease in width of the cortex due to diminution in size of the cells of the zonae fascicularis and reticularis (Fig. 1). Cytoplasmic lipid (Fig. 7) and birefringent material (Fig. 11) were also largely lost. The glomerulosa remained largely unchanged except for a possible slight widening, and the presence of coarse birefringent particles. M.A.D. in doses of 0.5 mgm. daily prevented these changes (Figs. 2, 8, and 12), and the adrenals of the animals presented a microscopic picture similar to that of uninjected controls. No microscopic changes were observed in the adrenals of rats treated with M.A.D. alone.

TABLE 3. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL UPON ADRENAL WEIGHT IN HYPOPHYSECTOMIZED RATS

Treatment	Mean body weight, g.		Mean adrenal weight
	Initial	Final	Mg. ±S.E.
I. Cortisone 2 mg. daily	175	125	13.79 ± .50
II. M.A.D. 3 mg. daily	180	168	15.50 ± .83
III. Cortisone + M.A.D.	177	136	20.85 ± .94
IV. Uninjected controls	174	160	15.44 ± .75

Effect of cortisone and methylandrostenediol upon hypophysectomized rats. Rats that were hypophysectomized about 4 days were divided into groups as shown in Table 3. This experiment was performed twice, but as the results were identical in the two experiments, the data have been combined in the table. Each figure in Table 3 represents the mean of 16 to 20 rats. Treatment was continued for 18 days. The dose of M.A.D. was higher than that found in the preceding experiment to be necessary for optimal effects. Indeed, probably not all the administered androgen was absorbed, as deposits were found at injection sites when the rats were sacrificed. However, sufficient M.A.D. was absorbed to have a pronounced effect in combination with the cortisone.

None of the treatments succeeded in preventing the loss in body weight following hypophysectomy. Indeed, those treated with cortisone lost significantly ($P<0.01$) more than did the untreated controls and treat-

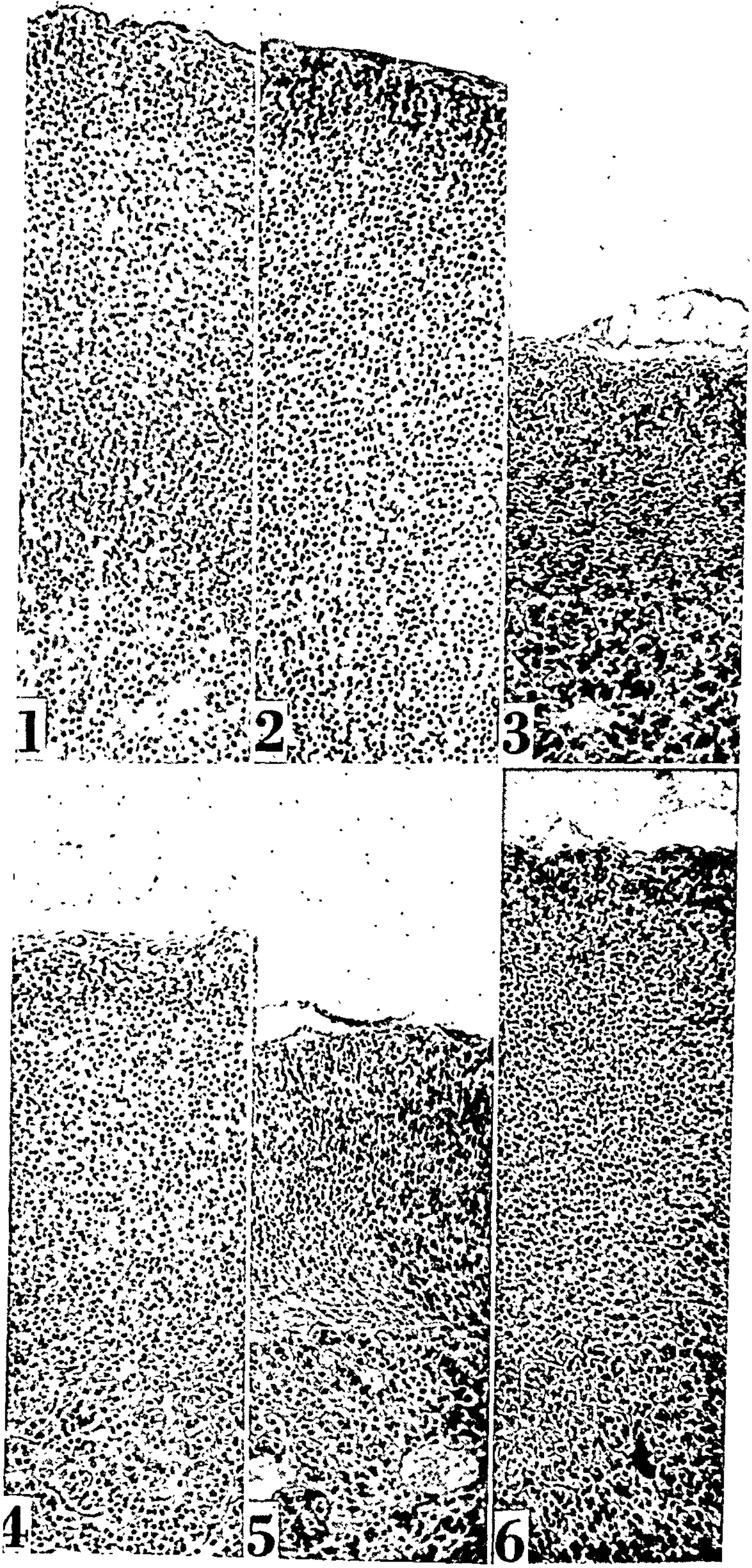

PLATE I

ment with the androgen did not prevent this excessive weight loss (P for difference in body weight loss, Groups I and III >0.05). M.A.D. alone did not significantly alter the weight loss in comparison with uninjected *controls* ($P>0.05$). *Therefore, only the cortisone affected weight loss in the* hypophysectomized rats; this loss in weight was greater than that commonly seen in intact rats treated with a similar dose of cortisone.

Effects on adrenal weights were very different from effects on body weight. Neither cortisone nor M.A.D. restored the adrenals of the hypophysectomized rats, but the combination of the two steroids restored the adrenals almost to the size of those observed in intact rats of comparable body weights.

The atrophy of the adrenals in the hypophysectomized rats receiving cortisone is shown in Figure 3, while Figure 4 demonstrates the repair occurring as a result of combination therapy of the two steroids. The atrophy in the presence of cortisone alone was not significantly greater than *that seen in uninjected hypophysectomized controls, in confirmation of* Stebbins (1951b). The extremely small size of the cells of the inner zones of the cortex of these atrophic glands (Fig. 3) was due to loss of cytoplasm, particularly of cytoplasmic lipid; the glomerulosa, in contrast, was thickened and showed a heavy deposition of lipid (Fig. 9). M.A.D. alone did not effectively restore the normal histological picture. The cells of the inner zones in these rats contained large vacuoles in which no lipid could be demonstrated. Considerable repair of the adrenal cortex could be seen in

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PLATE I. PARAFFIN SECTIONS OF RAT ADRENALS ×108. HEMATOXYLIN-EOSIN

FIG. 1. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. The cells of the inner cortical zones are reduced in size due to loss of cytoplasmic lipid. The glomerulosa appears slightly wider than normal.

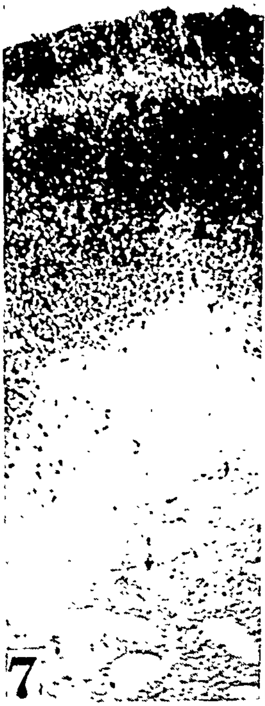
FIG. 2. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. methylandrostenediol for 18 days. Inner cortical cells contain more cytoplasmic lipid than in Fig. 1. The glomerular zone is essentially normal.

FIG. 3. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. Atrophic cells of inner cortex have lost lipid and nuclei are small and closely packed. Glomerulosa appears thickened.

FIG. 4. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Cells of zonae fascicularis and reticularis are larger and contain more cytoplasmic lipid and the glomerulosa is thinner than in Fig. 3.

FIG. 5. Intact rat injected daily with 2.5 mg. of cortisone acetate for 32 days. The section gives evidence of severe contraction of inner cortex with virtual extinction of cellular lipid and small closely arranged nuclei. Glomerulosa appears thickened.

FIG. 6. Intact rat injected with 2.5 mg. of cortisone acetate for 31 days and 0.5 mg. of methylandrostenediol daily from 18th to 31st day. Section displays a much wider inner cortex due to presence of lipid and pink-staining cytoplasm. Subglomerular and outer fascicular cells appear more closely packed than cells deeper in cortex.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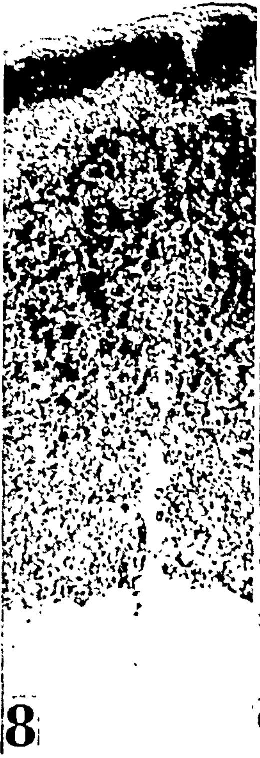
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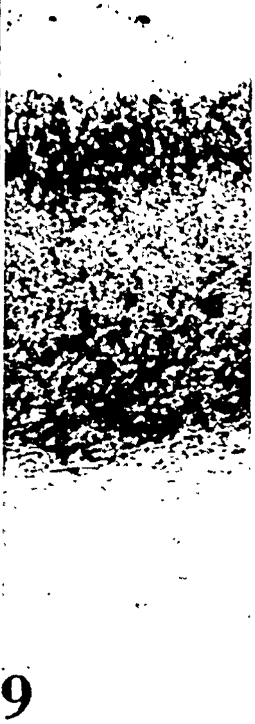
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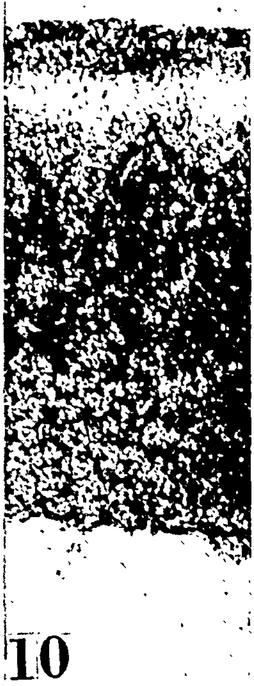
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PLATE II

the animals receiving combined therapy with the two steroids (Fig. 4). The adrenal cortices of these animals were much wider than those of untreated controls, or of those receiving either steroid alone. The cells were larger and more distinct and contained considerably more lipid (Fig. 10), while the birefringent particles were finer and more diffusely arrayed (cf. Figs. 13 and 14). In these respects the glands more nearly resembled those of intact rats. In general, the histological study confirmed the data obtained on adrenal weights.

Reversal of cortisone-induced adrenal atrophy. Having determined that M.A.D. could prevent cortisone-induced atrophy, and that the combination of steroids was effective in hypophysectomized rats, we next attempted to determine whether M.A.D. could restore the adrenals of intact rats in which atrophy had already been produced by cortisone, and also whether M.A.D. could hasten recovery of the adrenals after discontinuance of cortisone injections. Accordingly, 78 male rats weighing about 95 grams were injected with 2.5 mg. of cortisone daily for 17 days. These animals were then divided into groups of six rats, as shown in Table 4. In some groups (Ia to Id) cortisone was continued for varying lengths of time to obtain control figures on the degree of atrophy produced by this steroid.

PLATE II. DISTRIBUTION OF LIPID AND BIREFRINGENT MATERIAL IN FROZEN SECTIONS OF RAT ADRENALS × 108. SUDAN BLACK B.

FIG. 7. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Shrunken cells of inner cortex have lost appreciable amount of lipid.

FIG. 8. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Larger cortical cells are laden with lipid.

FIG. 9. Hypophysectomized rat treated with 2.5 mg. of cortisone acetate for 18 days. Lipid is lost from outer area of inner cortex. Thickened glomerulosa contains large amount of cellular lipid.

FIG. 10. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Large cells of fasciculata and reticularis are laden with lipid. Thinner glomerulosa shows less lipid than in Figure 9.

FIG. 11. Intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Coarse birefringent accumulations appear in glomerulosa with very fine particles in inner cortex. Subglomerular zone between them is empty.

FIG. 12. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of of methylandrostenediol. Fine birefringent particles appear in the glomerulosa. A mixture of fine and coarse particles are seen in the fasciculata and reticularis.

FIG. 13. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. The glomerulosa displays closely arranged fine particles. The atrophic cells of inner cortex contain for the most part very fine delicate birefringent particles.

FIG. 14. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Very fine particles of glomerulosa are more freely distributed than in Figure 13. Larger cells of inner cortex contain larger and more distinct birefringent material.

TABLE 4. REVERSAL BY METHYLANDROSTENEDIOL (M.A.D.) OF ADRENAL ATROPHY PRODUCED BY CORTISONE ACETATE

Treatment	Mean adrenal weight, mg.
I. Cortisone acetate 2.5 mg. daily for:	
a. 17 da.	11.2
b. 21 da.	11.5
c. 24 da.	11.7
d. 31 da.	10.6
II. Cortisone acetate 2.5 mg. daily for:	
a. 21 da., M.A.D. 0.5 mg. last 4 da.	15.0
b. 24 da., M.A.D. 0.5 mg. last 7 da.	14.2
c. 31 da., M.A.D. 0.5 mg. last 14 da.	15.4
III. Cortisone acetate 2.5 mg. for 17 da.:	
a. sacrificed 4 da. after last injection	16.3
b. sacrificed 7 da. after last injection	23.7
c. sacrificed 14 da. after last injection	27.7
IV. Cortisone acetate 2.5 mg. for 17 da., then cortisone discontinued and M.A.D. 0.5 mg. started:	
a. sacrificed 4 da. after last cortisone	17.6
b. sacrificed 7 da. after last cortisone	21.6
c. sacrificed 14 da. after last cortisone	25.4

In comparable groups (IIa to IIc) M.A.D. was given in conjunction with cortisone during the last portion of the period of cortisone treatment. Additional groups were studied to determine the rate of adrenal recovery after discontinuance of cortisone without (IIIa to IIIc) and with (IVa to IVc) M.A.D.

The data demonstrate that after cortisone had been injected for 17 days, no further adrenal atrophy took place when injections were prolonged (Groups Ia to Id). When adrenal atrophy was allowed to occur, and then M.A.D. was given along with continuing cortisone injections (Groups IIa to IIc), a significant degree of recovery occurred ($P=0.02$). This recovery did not completely restore the adrenals to normal size, however. After cortisone was discontinued, recovery of the adrenals took place at the same rate whether or not M.A.D. was administered during the recovery period (Groups III and IV); in each case, the difference between adrenal size in the "recovery" animals with and without M.A. D. was not significant ($P>0.05$).

The histological picture of adrenal repair shown in Figure 6 is taken from an animal of Group IIc. This is in contrast to the markedly atrophic adrenals of the animals which received cortisone for the same length of time without M.A.D. (Fig. 5, animal of Group Id). Recovery of the adrenal cortex after discontinuance of cortisone has been described in a previous communication (Winter, Silber and Stoerk, 1950). Essentially, the same restoration of the histological picture was seen in the present series. Histologically, there was not much difference between the recovery with and without M.A.D., although there seemed to be a slight difference in favor of the M.A.D.-treated rats after 4 days of recovery

TABLE 5. EFFECT OF CORTISONE ACETATE AND METHYLANDROSTENEDIOL (M.A.D.) UPON THE EDEMA PRODUCED BY INJECTION OF IRRITANT (0.1 CC. OF 0.5 PER CENT $AgNO_3$ SOLUTION IN RAT'S FOOT)

Treatment	Amount of edema*	
	3 hours	24 hours
Untreated controls	51.8	42.2
M.A.D.—3 mg. daily for 3 da.	42.5	48.7
Cortisone—1.5 mg. daily for 3 da.	33.8	30.8
M.A.D.—3 mg. +cort. ac. 1.5 mg. daily for 3 da.	25.0	27.2

* "Amount of edema" = difference in cross-sectional area of foot before and after irritant (see text).

(Group IVa compared to IIIa) and a slight difference in favor of those without M.A.D. in the later recovery groups (IVb and c compared to IIb and c). These differences were too small to be certain of their significance.

Effect of methylandrostenediol upon the anti-inflammatory action of cortisone. Since M.A.D. clearly antagonized the production of adrenal atrophy by cortisone, it was of interest to determine whether this androgen would also antagonize the anti-inflammatory effects of cortisone. Sely (1949) has shown that cortisone inhibits the local effect of irritants injected under the plantar surface of the rats' foot. We have employed a similar technique, using as an irritant 0.1 cc. of 0.5 per cent $AgNO_3$ solution, and measuring the edema 3 hours and 24 hours after the irritant. The animals were pretreated for 3 days with steroids subcutaneously. The results are given in Table 5. The "amount of edema" was calculated as follows: measurements of two diameters of the rat's foot were made by calipers at about the mid-metacarpal line. Assuming the cross-section of the foot to be an ellipse, the cross-sectional area in sq. mm. was calculated. The difference between this area before and after injection of irritant was termed "amount of edema."

TABLE 6. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL (M.A.D.) UPON GRANULOMA FORMATION INDUCED BY SUBCUTANEOUS COTTON PELLETS IN RATS

Treatment	Per cent increment in dry weight cotton pellet
A. Systemic steroids	
Untreated controls	146 ±6.6*
M.A.D. 3 mg. daily for 6 da.	123 ±9.8
Cortisone acetate 2 mg. daily for 6 da.	70 ±5.6
Cortisone acetate 2 mg. +M.A.D. 3 mg. daily for 6 da.	70 ±3.5
B. Local Steroids	
Cortisone acetate 0.25 mg.	123†
Controls	162
M.A.D. 0.75 mg.	143
Controls	143
Cortisone acetate 0.25 mg. +M.A.D. 0.75 mg.	98‡
Controls	139

* Standard error.
† Difference between controls and treated animals, $P = 0.05$.
‡ Difference between controls and treated animals, $P < 0.01$.

It is apparent that M.A.D. alone had no effect upon the production of edema, and that cortisone was not less effective in presence of the androgen.

In another experiment, cotton pellets were implanted subcutaneously and the amount of granuloma tissue formed at the site of the pellet was determined by removing the pellet together with the granuloma around it after 6 days, according to the technique described by Meier, Schuler and Desaulles (1950). This reaction is inhibited by cortisone, either administered systemically or by local application (incorporation of the steroid in the pellet). In the test of the local action of the steroid, a control pellet is placed on one side of the animal, and a pellet containing steroid on the opposite side. Thus, each animal serves as its own control. Table 6 gives the results by both methods of administration. It is evident that M.A.D. does not have any influence upon the amount of granuloma formed, nor does it antagonize the inhibition of the reaction by cortisone.

Both these experiments make it clear that although M.A.D. antagonizes the effect of cortisone in producing adrenal atrophy, it does not antagonize the anti-inflammatory action of cortisone.

DISCUSSION

Although cortisone has proven to be useful in a variety of conditions, it exerts certain hormonal effects which do not contribute to its therapeutic value, and which, indeed, it would be desirable to avoid, if possible. Among these is adrenal atrophy. This investigation was undertaken with the thought that if one or more of these unwanted effects could be counteracted without diminishing the desired actions of the hormone, the usefulness of cortisone might be enhanced. This hope seems to have been at least partially realized in these animal experiments, although the therapeutic applicability of these results has not as yet been demonstrated. The hyperadrenocortinism produced in patients by large doses of cortisone resembles in many respects the findings in Cushing's syndrome. Albright (1942–43) found androgens to be of value in correcting many of the manifestations of this disease. Pearson (1951) also reported that testosterone would counteract some of the effects of cortisone on normal tissue.

Both testosterone and methylandrostenediol exerted a sparing action upon the adrenal cortices of animals treated with cortisone. Both these compounds are androgens, and both also have anabolic action as shown by growth-promotion and nitrogen balance tests (Kochakian, 1949; Homburger *et al.*, 1950). Methylandrostenediol, however, has been reported to be relatively high in the anabolic action typical of androgens, but low in androgenic potency (Gordan *et al.*, 1951). It is possible that the sparing action of these steroids on the adrenal cortex of cortisone-treated rats is a consequence of their anabolic action. In the present experiments, no such action could be demonstrated upon the growth-suppressing action of cortisone. However, it is not necessary to assume that steroids have a qualitatively similar action upon metabolic phenomena in all the tissues of the body; indeed, there is good evidence that cortisone has opposite metabolic effects upon different tissues (Kochakian and Robertson, 1951). It is therefore not unreasonable that our experiments might show an anabolic action of M.A.D. on the adrenals even though they did not demonstrate such action on the body as a whole.

In the intact animals, it might be argued that M.A.D. could act as a stress to effect the release of ACTH, and adrenal atrophy could be prevented by release of this hormone from the hypophysis. Alternatively, M.A.D. *might block the inhibiting effect of cortisone upon the release of* ACTH. The results in hypophysectomized rats deny both these alternatives. The repair of adrenals after hypophysectomy when both cortisone and M.A.D. are used, but not when either is administered alone leads to the surprising conclusion that both cortisone and the androgen are necessary for this result. Also, in the experiments on adrenal repair after establishment of adrenal atrophy by cortisone, M.A.D. brings about some repair if it is administered while cortisone is being given. On the other hand, when M.A.D. is given alone; i.e., after discontinuance of cortisone, adrenal repair does not proceed more rapidly than if no steroid is being given. Therefore, in this experiment as in the experiment with hypophysectomized rats, it appears that it is the combination of the two steroids which is effective against adrenal atrophy, rather than either one alone. On the other hand, no combination of the two steroids was used which gave adrenals larger than those of nomal intact rats (several dosage ratios were tried which were not included in the tables) nor did M.A.D. alone in intact rats produce large adrenals.

SUMMARY

1. Testosterone propionate or methylandrostenediol administered in conjunction with cortisone acetate in rats prevented the adrenal atrophy which occurred when cortisone alone was given. Adrenal cortices of rats given both the androgen and cortisone were essentially normal in size and histological appearance.

2. Combination therapy with methylandrostenedial and cortisone produced considerable repair of the adrenals of hypophysectomized rats. Neither steroid alone produced repair of adrenals in such animals.

3. Adrenals made atrophic by cortisone injection could be partially repaired by administration of methylandrostenediol while cortisone treatment was continued. However, adrenal recovery after discontinuance of cortisone was not hastened by injection of the androgen.

4. Methylandrostenediol administered with cortisone did not inhibit the anti-inflammatory action of cortisone.

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GONADOTROPINS OF THE PITUITARY GLAND AND THE URINE OF THE ADULT HUMAN FEMALE

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IN A previous paper (Bahn *et al.*, 1952), a study of the gonadotropins of the pituitary gland and the urine of the adult human male was made. The purpose of this paper is to present similar data on the pituitary gland and the urine of the adult human female.

I. MATERIALS AND METHODS

A. Animals. Male and female Wistar rats (30 to 40 days old) were hypophysectomized; injected, some with pituitary homogenate and others with urine concentrate; and subsequently studied as described previously.

B. Pituitary tissue. The pituitary gland of 2 adult human females was obtained within three to four hours following death. Neither of the patients presented any obvious endocrinopathy. Menstruation had occurred at regular monthly intervals. The ages of the patients were 38 and 46 years. There was no prolonged terminal illness. The combined wet weight of the anterior lobes of the pituitary glands was 1,219 mg.

C. Urine. A number of complete twenty-four hour specimens of urine were obtained from 5 healthy menstruating adult females during all phases of the menstrual cycle.

II. RESULTS

A. Pituitary homogenate in female animals. The results observed in hypophysectomized female rats receiving anterior pituitary homogenate are listed in Table 1. The data indicate that both follicle-stimulating (FSH) and luteinizing (LH, ICSH) hormones are present in the normal adult human female hypophysis. Neither early follicular growth nor repair of the ovarian interstitial "deficiency cells" was observed until a total dose of 3 mg. of the pituitary homogenate was given.

Uterine and ovarian enlargement occurred at a dose of 10 mg. The increase in ovarian weight was associated with the presence of many large cystic follicles. There was only slight luteinization of the granulosa cells while the interstitial and thecal cells had undergone marked lutein cell transformation. The response to 30 mg. of anterior pituitary homogenate

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TABLE 1. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals			Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	4	0	5.6	0.10	15.0	0.28	0	0	D	0
Intact	6	0	18.6	0.18	118.3	1.17	+++	+	R	+
Hyp.	4	1	8.0	0.12	21.8	0.32	0	0	D	0
Hyp.	3	3	9.2	0.14	22.9	0.35	+	0	R	0
Hyp.	4	10	38.8	0.54	98.8	1.37	++++	0	L	0
Hyp.	1	30	81.2	1.35	78.4	1.31	++++	+	L	+

Abbreviations: Hyp. = Hypophysectomized; mg./gm. = Organ weight per gram body weight; D = Deficient; R = Repaired; I.T. = Interstitial tissue; 0 = absent; + = Present; L = Luteinized; V.O. = Vaginal opening; F.D. = Follicular development.

was characterized by additional ovarian enlargement and the formation of numerous corpora lutea.

In terms of the previously defined units, there is approximately 1 unit of FSH and 1 unit of LH per 3 mg. of pituitary homogenate, or a total of about 200 units of both hormones per pituitary of the adult human female.

B. Pituitary homogenate in male animals. Table 2 lists the results on hypophysectomized male rats. At the 3 mg. dose level FSH activity was indicated by a doubling of testicular weight, an increase in tubular diameter and active spermatocytic proliferation as compared with the controls. Thirty milligrams of pituitary homogenate tripled the testicular weight, but did not completely restore the normal testicular histologic pattern. Only the testis of the intact control animals exhibited spermatozoa.

TABLE 2. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals			Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	2	0	13.9	0.22	15.0	0.23	47.3	0.74	0.10	+	0	0	U.
Intact	5	0	62.2	0.58	41.0	0.38	132.2	1.22	0.22	++++	+	+	Di.
Hyp.	3	1	27.3	0.39	22.5	0.32	75.6	1.09	0.14	++	0	0	U.
Hyp.	3	3	45.8	0.64	41.1	0.57	115.4	1.61	0.16	+++	0	0	Di.
Hyp.	2	10	54.2	0.77	46.5	0.66	140.1	2.00	0.15	+++	0	0	Di.
Hyp.	3	30	52.7	0.68	60.1	0.78	163.9	2.13	0.16	++++	+	0	Di.

Abbreviations: M.T.D. = Mean tubular diameter (millimeters); S.A. = Spermatocytic proliferation; St. = Spermatid; Sz. = Spermatozoa; I.T. = Interstitial tissue; U. = Undifferentiated; Di. = Differentiated; Others = See table 1.

At the 3 mg. dose level LH activity was indicated by the appearance of well-differentiated Leydig cells. Nevertheless, indirect evidence of Leydig cell function was present at the 1 mg. dose level as judged by the weights and results of histologic examination of the prostates and seminal vesicles. The amount of homogenate necessary to repair the deficient Leydig cells as judged by histologic evidence was 3 mg. The same amount also brought about a distinct response in the tubular apparatus of the testis as judged by testicular weight, tubular diameter and spermatogenic activity. Again, roughly equal FSH and LH activities, about 200 units of each, are present in the adult female pituitary.

C. Urine in female animals. Table 3 shows the effect of a concentrate equivalent to 1,200 cc. of pooled urine from adult females. The presence of FSH was shown by the increased ovarian weight and the marked response of the ovarian follicles. The latter were large cystic structures without signs of luteinization. Ova were present within these follicles. Both ovarian and uterine enlargements were about equal to that brought about by 3 to 10 mg. of pituitary homogenate.

TABLE 3. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals				Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	2	0	0	7.3	0.11	16.3	0.23	0	0	D	0
Hyp.	3	+	0	8.7	0.12	16.3	0.23	0	0	D	0
Hyp.	5	+	+	20.2	0.30	76.8	1.15	++++	0	D	+
Intact	5	0	0	18.4	0.16	83.9	0.73	+++	+	R	+

Abbreviations: Same as in table 1.

The presence of LH was shown by the copious secretion of estrogen, resulting in uterine enlargement. However, since there was no repair of the interstitial tissue of the ovary, less than 1 unit of LH as previously defined was present in 1,200 cc. of urine.

D. Urine in male animals. Table 4 lists the weights of organs of hypophysectomized male rats receiving urinary concentrate. Little change occurred in the number or the size of the interstitial cells as compared to the hypophysectomized control animals. Nevertheless, the weight of the ventral prostate, epididymis and seminal vesicles was distinctly increased over that of the hypophysectomized controls. Moreover, microscopic examination of the ventral prostate and seminal vesicles gave evidence of

TABLE 4. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals				Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	4	0	0	6.5	0.09	8.6	0.12	49.5	0.69	0.13	+	0	0	U.
Hyp.	5	+	0	6.6	0.10	8.9	0.14	41.9	0.66	0.13	+	0	0	U.
Hyp.	5	+	+	9.4	0.14	10.9	0.16	76.0	1.09	0.16	+++	+	0	U.
Intact	5	0	0	65.7	0.55	44.3	0.37	171.3	1.44	0.23	++++	+	+	Di.

Abbreviations: Same as in Table 2.

IN VITRO BIOASSAY OF CORTICOTROPHIN

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WE HAVE observed that pituitary adrenocorticotrophic hormone (corticotrophin), added *in vitro,* stimulates the formation of corticoids by isolated rat adrenals and that the stimulation is approximately proportional to the amount of corticotrophin added (Saffran, Grad and Bayliss, 1952). This observation has been adapted to the bioassay of corticotrophin.

METHOD

Four adult Sprague-Dawley rats, of either sex, are used for each assay. The adrenals are prepared as previously described (Saffran, Grad and Bayliss, 1952). Care must be taken to keep the pair of adrenals from each rat together during the preparation. Four adrenals, one from each pair, are weighed together, bisected, and placed into a Warburg vessel containing 2 ml. of the Krebs-Ringer-phosphate-glucose medium. The remaining four adrenals are then weighed, bisected, and placed into a second Warburg vessel. The flasks are attached to Warburg manometers, flushed with oxygen, and are shaken at 38° for 1 hour. At the end of this period (the preincubation period) the media from both flasks are removed with suction and discarded. Both flasks are then recharged with fresh medium, and a small volume (about 0.1 ml.) of the standard corticotrophin solution is added to one flask and the unknown corticotrophin solution to the other. Approximately the same activity of corticotrophin should be added to both flasks. The flasks are again flushed with oxygen and shaken at 38°, this time for 2 hours. At the end of this period the media are analyzed separately for corticoids by a slight modification of the method previously described (Saffran, Grad and Bayliss, 1952). The modification consists of passing the chloroform extract through filter paper instead of drying with sodium sulfate. The filtered chloroform extract is then taken to dryness as previously described.

The *amount* of corticotrophin is expressed as units of the standard preparation and mg. of the unknown per 100 mg. of adrenal tissue in the flask. The *activity* of the corticotrophin is expressed as μg. of corticoids produced by 100 mg. of adrenals in 2 hours.

The potency of the unknown sample is calculated as follows:

$$\text{Potency (units per mg.)} = \frac{\text{units of standard corticotrophin x activity of unknown}}{\text{mg. of unknown corticotrophin x activity of standard}}$$

The procedure is outlined in the sample protocol. In actual practice several levels of standard and unknown preparations are matched for each determination of potency.

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SAMPLE PROTOCOL

Comparing Armour's corticotrophin with the U.S.P. reference standard

	Standard	Unknown
Weight of 4 adrenals, mg.	48	45
Tissue added to flask	9:58 A.M.	10:00 A.M.
Preincubation period	10:10 to 11:10 A.M.	
Corticotrophin added	U.S.P.	Armour
ml.	0.1	0.1
units or mg./flask	0.025 unit	0.012 mg.
units or mg./100 mg.	0.053 unit	0.027 mg.
Second incubation period	11:30 A.M. to 1:30 P.M.	
Observed optical densities:		
225 mμ	0.285	0.285
240 mμ	0.260	0.254
255 mμ	0.174	0.167
Calculated O.D.	$.260-\frac{.285+.174}{2}=0.031$	$.254-\frac{.285+.167}{2}=0.028$
Total μg. corticoids	5.6	5.1
μg. corticoids/100 mg.	11.75	11.3

$$\text{Potency of corticotrophin, Armour}=\frac{0.053\times 11.3}{0.027\times 11.75}=1.93\text{ units/mg.}$$

EXPERIMENTAL

Extraction of corticoids

A methanolic solution of the residue from a chloroform extract of the medium alone absorbs light in the ultraviolet at the wave lengths used to measure the corticoids (Fig. 1). Although the absolute value of the blank absorption varies somewhat, the shape of the curve is almost invariably the same. Because the blank curve approaches a straight line it is possible to use the calculation of Allen (1950) to estimate the absorption at 240 mμ caused by the α,β-unsaturated ketonic grouping of the corticoids. The absorption curve of a chloroform extract of the incubation medium of rat adrenals kept at 2° for 2 hours in the presence of corticotrophin is very similar to the blank. Figure 1 includes the curve of an extract of the incubation medium of adrenals kept at 38° with corticotrophin present and the absorption curve of cortisone for comparison with the blank curves.

Attempts to eliminate the high blank absorption by replacing the chloroform with methylene chloride, ethyl acetate, heptane, or ether failed because these solvents either yielded higher blank values than chloroform or because they did not extract the corticoids from an aqueous medium.

Preincubation

In this paper "preincubation" means the incubation of the adrenals for

Rate of corticoid formation

The rate of formation of corticoids, in the presence or absence of added corticotrophin, is approximately constant for 4 or 5 hours (Fig. 3).

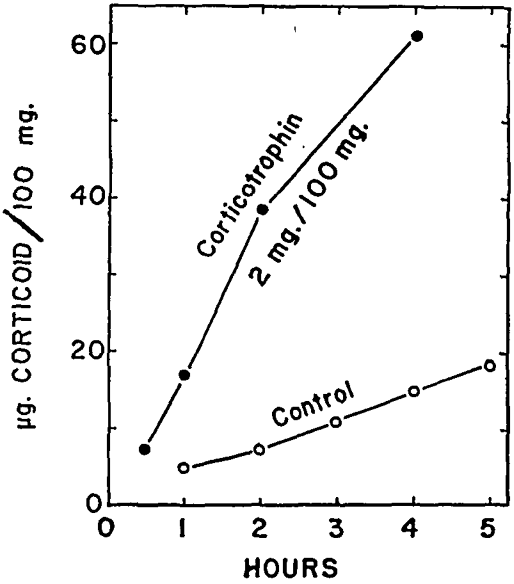

FIG. 3. Rate of formation of corticoids by bisected rat adrenals in the presence and absence of added corticotrophin.

Persistence of corticotrophin effect

Rat adrenals, incubated for 1 hour with corticotrophin, produce increased amounts of corticoids even after two thorough rinsings with the medium (Fig. 4).

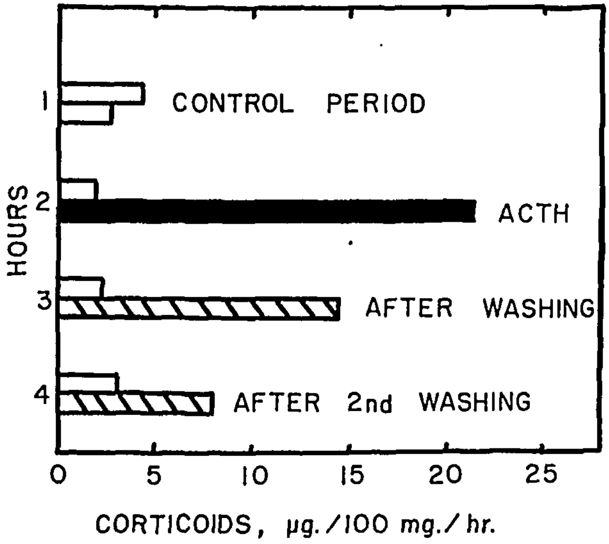

FIG. 4. Persistence of the effect of added corticotrophin after washing of the adrenal tissue.

Incubation in blood

Because of the reported greater production of corticoids by beef adrenals when perfused with whole blood than with plasma (Hechter *et. al.*, 1951), bisected rat adrenals were incubated in citrated homologous blood instead of the usual medium. In this experiment a mixture of 5% carbon dioxide in oxygen was used as the gas phase. At the end of 2 hours little or no corticoids were found in a chloroform extract of the blood (Table 3).

TABLE 3. EFFECT OF CHANGES IN CONDITIONS OF INCUBATION ON THE FORMATION OF CORTICOIDS

Conditions	Corticotrophin added	Corticoids formed	Oxygen consumption
	units/100 mg.	μg./100 mg. per 2 hrs.	μM/100 mg. per 2 hrs.
Citrated blood and 5% CO_2	0	4.1	
	1.7	3.4	
	0	0	
	1.7	9.4	
	0	0	
	1.6	0	
Oxygen	1.7	32.1	12.5
Air	1.7	23.9	3.9
Nitrogen	1.6	0	0
	1.7	0	0
Bisected	1.5	24.8	11.6
	1.7	30.0	12.2
Minced	1.5	28.9	7.6
	1.6	21.6	7.2
Homogenized	0	0	1.0
	1.0	0	1.2

N.B. These samples were *not* preincubated.

Oxygen requirement

The oxygen requirement of the system that forms corticoids was investigated by incubating rat adrenals in air and in nitrogen. The formation of corticoids was somewhat lower in air than in oxygen, but no corticoids were formed during the anaerobic incubation in nitrogen (Table 3).

Mincing and homogenization

Mincing the adrenal tissue with scissors does not affect the formation of corticoids, but there is no formation in an unfortified homogenate of adrenals (Table 3).

Agreement between duplicates

Four adrenals, one from each of four rats, form about the same amount of corticoids as the four remaining adrenals from these animals, when the two lots are incubated under the same conditions (Table 4).

TABLE 4. AGREEMENT BETWEEN DUPLICATE INCUBATIONS

	Corticotrophin added	Corticoids formed	
	units/100 mg.	µg./100 mg./2 hrs.	
Not preincubated	0	4.2	2.6
	0	1.6	1.9
	0	3.3	4.2
	1.0	12.6	9.9
	2.5	18.5	16.0
	2.6	20.3	16.1
Preincubated	0	5.8	4.6
	0	3.8	7.4
	0	3.0	1.5
	0.8	32.3	35.8

RESULTS

The *in vitro* bioassay method was used to determine the potency of several preparations of corticotrophin. The values agree very well with the estimated potencies based on the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) (Table 5).

TABLE 5. POTENCY OF CORTICOTROPHIN PREPARATIONS BY THE *in vitro* BIOASSAY

Preparation	Estimated potency by *in vitro* assay	Claimed potency*
	U.S.P. units/mg.	U.S.P. units/mg.
Armour, lot J16609	1.6	2
	1.9	
	1.9	
Astwood, crude	1.6	2
	2.0	
	1.8	
Astwood, purified	50.1	60–100
	52.6	(average, 80)
	56.2	
	66.6	
Canada Packers, lot 2-C	3.1	3–4
	2.7	
	3.5	
Canada Packers, lot FU	3.0	3–4
Canada Packers, lot I	2.9	4–5
(deteriorating sample because of high moisture content)	2.7	

* Estimated by the method of Sayers, Sayers and Woodbury (1948). Values supplied by the donors of the corticotrophin preparations.

DISCUSSION

Although the optimum conditions for the *in vitro* assay method have not been established yet, it yields results that agree very well with the adrenal ascorbic acid depletion method. The *in vitro* method has several advantages. Firstly, the same animals are used to provide tissue for both the standard and unknown preparations of corticotrophin. Secondly, there is no need to maintain a supply of hypophysectomized animals because in the *in vitro* assay the adrenals are effectively removed from the influence of the pituitary. Thirdly, a known function of corticotrophin, namely, the control of the secretion of adreno-cortical hormones, is used as the basis of the assay. A disadvantage of the method is its relative insensitivity, but this is of little consequence when the potency of a preparation of corticotrophin is being determined.

The method is based on an assumption for which there is no direct evidence. It is, that a given group of adrenals, incubated together, will produce an amount of corticoids that is proportional to the amount of corticotrophin added. In the case of the adrenals (Fig. 2) that were not preincubated, the response to increasing amounts of corticotrophin seems to be more or less linear. After preincubation, the adrenal response curve is very steep, but for the assay it is assumed to be linear as well. That this assumption is valid to some extent is shown by the good agreement among replicate assays of the various preparations of corticotrophin (Table 5).

Both adrenals of a rat respond similarly to corticotrophin (Table 4). This allows the comparison of standard and unknown preparations of corticotrophin on similarly-reacting tissue. If homogeneous preparations of adrenal tissue can be shown to respond to corticotrophin like bisected adrenals, it would be possible to divide the tissue into more than two aliquots and allow the comparison of more than two samples using the same tissue. That this might be so is indicated by the response of an adrenal mince to corticotrophin (Table 3).

Preincubation increases the sensitivity of the adrenals to corticotrophin about 20–25 times. During the preincubation period a large proportion of the ascorbic acid is leached from the adrenal tissue (Saffran, Bayliss and Webb, 1951) and the tissue may have had the time to recover from any previous stimulation by corticotrophin in the animal. However, there is, as yet, no evidence for any explanation of the effect of preincubation.

The effect of corticotrophin on the adrenal *in vitro* persists after washing the tissue (Fig. 4). This observation is similar to that of Stadie and his group (Stadie, Haugaard and Marsh, 1951) on the *in vitro* effect of insulin. It is not possible now to state whether the corticotrophin is "bound" by the adrenal tissue.

SUMMARY

The stimulation by corticotrophin of the production of corticoids by bisected rat adrenals *in vitro* has been adapted to the assay of corticotrophin. The sensitivity of the method is greatly increased by the preincubation of the adrenal tissue without added corticotrophin for 1 hour, prior to exposure to corticotrophin. The range of the assay is approximately 0.04 to 0.1 U.S.P. units of corticotrophin per 100 mg. of adrenal tissue.

There is very good agreement between the *in vitro* bioassay and the adrenal ascorbic acid depletion method.

The stimulation by corticotrophin of the production of corticoids by rat adrenals persists after washing the tissue to remove the corticotrophin.

Corticotrophin stimulates the production of corticoids in an atmosphere of air as well as in oxygen, but the rat adrenal does not form corticoids in nitrogen.

An adrenal mince responds to added corticotrophin like bisected adrenals, but unfortified homogenates do not produce corticoids.

Acknowledgments

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NEUROHUMORAL CONTROL OF THE PITUITARY IN THE FOWL

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IN THE domestic fowl approximately 23 to 28 hours elapse between ovulation and the laying of the finished egg. During the intervening period the ovum acquires albumen in the magnum and shell membranes in the isthmal portion of the oviduct. This is accomplished in 4.5–5.0 hours and the remaining time is spent in the shell gland where the calcifierous shell is deposited. Warren and Scott (1935) showed that ovulation does not usually occur while there is an egg in any portion of the oviduct. These facts and the finding that, in hens laying daily, ovulation takes place within 30–60 minutes after oviposition, led to the assumption that an intricate timing mechanism may be involved to synchronize reproductive events in chickens. An observation in this laboratory that injury to the oviduct led to a profound upset in the laying cycle of hens caused more intensive analysis of the relationship between events in the oviduct and the endocrine function of the pituitary.

Rothchild and Fraps (1949) have shown that the ovulating hormone (LH) is released about 4 to 8 hours prior to ovulation. Thus, factors which may be involved either in stimulating or in inhibiting release of LH must exert their effects several hours prior to ovulation. Since there is only a relatively short interval between oviposition and the succeeding ovulation it becomes apparent that the events leading to ovulation usually occur while the egg is in the shell gland.

These observations led to the experiments reported here.[1] Initially, such irritants as bits of key chain, cork or pellets of paraffin were placed in the lumen of the magnum. These foreign bodies were attached to coarse surgical thread which in turn was passed through the wall of the oviduct and tied securely so as to prevent evacuation of the irritants from their original location in the lumen due to peristalitic movements of the oviduct. When paraffin pellets were used, they were soon softened by body

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[1] We wish to acknowledge the help of Dr. H. W. Norton in the design of some of the experiments and their statistical analysis. We thank Dr. H. W. McShan and R. K. Meyer (Univ. of Wisconsin) for the ISAP, Mr. Irby Bunding of Armour and Co. of Chicago for the LH and Dr. R. E. Waterman of the Schering Corp., Bloomfield, N. J. for the progesterone.

heat, torn loose from the thread fastening them to the oviduct wall and passed out. It was thus noticed that the thread alone, which remained after the paraffin pellet was evacuated, provided sufficient stimulus to cause profound changes in the laying cycle of these hens.

ANIMALS AND TECHNIQUES

The birds used were sexually mature hens less than two years old, of the White Leghorn and New Hampshire breeds. They were trapnested prior to the experiments to determine their normal rate of production. Only regularly laying birds were used. They were maintained in individual cages in laying batteries and allowed free access to a standard laying mash and water.

At the time of operation the birds were anesthetized with nembutal, and the oviduct was exposed through an incision in the abdominal wall. A loop of coarse surgical thread was passed through the wall of the oviduct into the lumen of the magnum in such a manner as to extend longitudinally through approximately an inch of the lumen. The thread was again passed through the wall of the magnum and the two ends of the thread were tied externally. In placing the loop care was taken to assure that the lumen of the oviduct was not obstructed.

In a second group of birds the oviduct was exposed in the same manner but no loop of thread was placed in the oviduct (sham operated controls). Birds in both groups were weighed at the start and at the end of the experiment; their combs were measured on alternate days and individual daily records of egg production were kept.

The hormones used in some of the experiments included progesterone in oil; a partially purified gonadotrophic preparation containing both follicle stimulating (FSH) and luteinizing hormones (LH) called ISAP; and a highly purified preparation of LH. The gonadotrophic hormones were dissolved in water and injected intravenously. One single injection of ISAP was made while the LH was injected twice daily until ovulation occurred. The progesterone was injected subcutaneously. Control animals were injected with equal amounts of oil or water.

The laid egg was the criterion used for determination of ovulation, with the exception of one experiment involving ISAP in which the hens were sacrificed and the number of ovulations verified at autopsy. At the conclusion of some of the experiments the birds were sacrificed, the ovaries and oviducts removed, weighed, measured and described with regard to their physiological condition.

After the basic physiologic effects on the ovary and the oviduct of the operation described were established, the hens were no longer sacrificed since the laid egg was taken as the evidence for success of the treatment when hormones were injected.

RESULTS

In order to establish the quantitative and qualitative effects of the loop on the laying cycle of the birds, on their reproductive organs and their comb size, groups of birds were killed 5, 10, 15, 20, 25 and 30 days after the operation. Eight of the birds destined to be killed at the different intervals in each group had a loop in the magnum, and eight served as sham-operated controls. Due to mortality at operation and during the course of the experiment some of the groups did not contain the full complement of eight birds at the time of autopsy (Table 1).

The most striking effect of the loop in the magnum was that it almost completely inhibited ovulation during the first 25 days after the operation. There were a few sporadic ovulations which were widely spaced (Table 1). *Eight of the 33 birds with a thread in the magnum laid a total of 12 eggs* (1.5 eggs per bird). That the operation itself, in absence of the loop of thread in the magnum, is not responsible for the interruption of laying is seen from the fact that during the same period 29 sham-operated hens laid 121 eggs (5.5 eggs per bird).

TABLE 1. EFFECT OF A THREAD IN THE MAGNUM ON THE SIZE OF OVARIES, OVIDUCTS AND COMBS, AND THE RATE OF OVULATION. OBSERVATIONS MADE AT 5-DAY INTERVALS

Treatment	Days from operation to autopsy	Number of birds	Number of birds laying	Total number eggs laid	Ovary weight (gms.)	Oviduct weight (gms.)	Comb area (cm.)	Average size of follicles (cm.)	Average number of follicles of ovulatory size (30 mm.)
L	5	6	1	2	45	53	25	19	0.75
			p =	0.15*	SE 8.7	6.2	5.1	1.4	
S	5	5	3	7	48	55	33	22	0.40
					8.0	2.0	5.6	1.0	
L	10	8	2	4	48	53	23	21	0.40
			p =	0.05*	5.9	4.1	1.6	0.8	
S	10	7	5	12	51	63	23	23	0.6
					3.5	4.3	4.0	1.1	
L	15	5	1	1	56	65	27	22	1.0
			p =	0.001*	5.3	3.5	1.4	1.3	
S	15	7	7	44	58	67	32	23	1.0
					3.4	3.3	3.8	1.1	
L	20	7	2	2	68	68	31	24	1.71
			p =	0.05*	4.8	3.7	4.4	1.0	
S	20	5	3	24	52	47	24	22	1.25
					10.9	5.9	2.0	1.4	
L	25	7	2	3	17	42	29	all atretic	0.00
			p =	0.01*	2.5	3.9	2.3		
S	25	5	4	34	43	30	25	22	1.12
					10.4	13.5	3.6	1.5	
L	30	6	3	31	59	69	30	—	—
			p =	0.06*	6.8	8.6	1.4		
S	30	6	5	55	52	60	34	—	—
					6.4	4.4	3.8		

L—hens, with thread in magnum of oviduct.
S—sham operated controls.
*—*levels of significance of differences in egg production between S and L.*
SE—standard error of means.

In all subsequent experiments essentially the same results were found as those discussed here. The presence of an irritant in the magnum modified the laying cycle of all hens treated, although not all the animals treated responded by complete cessation of laying. In most hens, laying was suppressed completely *(in 75% of the hens in one experiment, in 58% in* another). Most remaining hens laid occasional eggs, but few continued to lay at an almost normal rate. In most cases the rate of production was reduced very drastically as compared to the almost unimpaired rate of production of the sham-operated group.

The second important observation regarding the presence of the thread in the oviduct was that 30 to 40 days after the operation laying was gradually resumed and eventually became normal (Table 1, 30-day group).

It is also interesting that the loop affected neither the size, the morphological condition of the ovary, nor that of the oviduct or the comb (Table 1). Ova which were present in the ovary at the time of the operation seem to be maintained without degenerating during the first 20 days after the operation, thus accounting for the non-reduction in ovarian weight (Table 1). This is true not only for number of follicles but also for size. In this particular experiment atresia of follicles set in at about 25 days and was observed in all seven hens in this group. At 30 days, when the next group was killed, the ovaries were found to be normal and three of the six hens in this group were laying. Statistical analysis showed that the differences in production between the sham-operated and the hens with a thread in the magnum were significant in all groups except the 5-day and the 30-day groups (Table 1).

These observations led to the assumption that the irritant in the oviduct did not depress pituitary function because the size of the ovary (number and size of follicles) was not reduced for as long as 20 days after the operation. Since neither the ovary nor the oviduct or the comb were reduced in size, it appeared that the ovary continued to secrete androgen and estrogen at normal rates.

These facts led to the theory that the presence of an irritant in the uterus specifically prevented the release of sufficient amounts of LH from the pituitary to cause ovulation without, however, interfering with the other endocrine functions of the pituitary. The mechanism of this inhibiting action on the secretion of LH was thought to be neuro humoral in nature and experiments were conducted to test this assumption.

In order to establish the relationship of the presence of the thread to the inhibition of ovulation, two experiments were designed. In the first, 12 hens which had ceased laying after the placing of the thread were injected intravenously with a crude gonadotrophic preparation, ISAP, containing

TABLE 2. NUMBER OF OVULATIONS OCCURRING WITHIN 72 HOURS AFTER ONE INTRAVENOUS INJECTION OF 0.25 GRAM EQUIVALENT OF ISAP (ISAP 16: 43.6 MGS. EQUIVALENT TO 1 GRAM OF ACETONE-DRIED SHEEP PITUITARY POWDER)

Results	Number of hens
Did not ovulate	2
Ovulated 1 ovum	4
Ovulated 2 ova	4
Ovulated 3 and 4 ova	2

both FSH and LH. The frequency of induced ovulations was very high, 10 of the 12 hens ovulating while none of the controls responded (Table 2). The two injected birds which did not ovulate were noted to be in poor physical condition at the time of autopsy. In this experiment the birds were killed 72 hours after the injection of the hormone and the number of ova shed was determined at autopsy (Huston and Nalbandov 1950).

TABLE 3. EFFECT OF INTRAVENOUS INJECTIONS OF LH (ARMOUR) ON OVULATIONS OF HENS WITH THREAD IN THE MAGNUM. (HENS INJECTED TWICE DAILY UNTIL FIRST OVULATION)

Treatment	Dose of LH (mgs.)	Number of hens not ovulating	Number of hens ovulating	Number of days from first injection to ovulation
Thread in magnum*	0.0	6	2	—
Thread in magnum*	0.1	1	6	2, 2, 3, 3, 4, 7
Thread in magnum*	0.2	3	5	2, 4, 5, 6, 8
Sham operated†	0.0	1	6	—

* Had not ovulated from operation to first injection (9 days).
† Were in normal production during the whole period.

Since six of the ten hens ovulating in response to the ISAP injection shed more than one yolk, the dosage used was thought to be unphysiologically high. In the second experiment LH (Armour) was injected at two different dosages into two groups of hens of seven and eight animals respectively with threads in their magna. Both dosages of LH were effective in causing ovulations in the majority of the hens although the number of injections required to cause ovulations was much greater than in the case of ISAP (Table 3). The dosages of LH seemed within the physiological range since no more than one ovulation was produced in any of the treated hens.

It has been shown that release of LH from the pituitary can be induced in the fowl by progesterone (Fraps and Drury, 1943; Rothchild and Fraps 1949). Accordingly, the ability of progesterone to cause ovulation in hens with threads in the oviduct was assayed in a series of tests involving dosages of progesterone ranging from 0.3 to 6.0 mgs. In all cases a single

TABLE 4. ABILITY OF VARIOUS DOSES OF PROGESTERONE TO CAUSE OVULATION IN HENS WITH THREAD IN THE MAGNUM

Dose of progesterone (mgs.)	Number of hens not ovulating	Number of hens ovulating	Average number of ovulations per hen	Number of days from injection to ovulation
(oil)	6	0	0	—
0.3	3	0	0	—
0.6	3	0	0	—
0.9	3	0	0	—
1.0	3	0	0	—
2.0	2	2	1.00	2, 3
4.0	3	2	1.50	2, 5
5.0	3	4	1.25	1, 2, 2, 5
6.0	1	3	2.00	1, 2, 2

subcutaneous injection of the hormone was given. The results (Table 4) indicate that it is possible to cause ovulation with progesterone in birds in which the release of endogenous LH has been blocked by the presence of a loop of thread in the magnum. Apparently the progesterone levels required for ovulation in these hens are higher than those which had been found effective by Rothchild and Fraps (1949). These authors had established the egg-laying pattern of each hen accurately enough to predict within a few hours the expected time of maturation of a follicle. In the present experiments no such determination could be made, since laying had been interrupted by the placing of the irritant and most of the follicles of ovulatory size were quite old when the progesterone injections were made. The reduced effectiveness of progesterone observed in our experiments may be due to a loss of sensitivity of the "aged" follicles to LH or a reduced sensitivity of the pituitary to progesterone. This may explain the failure to induce ovulation in nine out of twenty hens injected with two or more milligrams of progesterone (Table 4).

During the course of these experiments it was noted that 20 to 40 days after the thread had been placed in the magnum, most of the operated birds gradually began to lay and eventually returned to normal laying frequency. This has been explained on the assumption that the neural mechanism involved becomes conditioned to the constant irritation of the oviduct and loses its ability to respond to the stimulus. To test this possibility two experiments were performed. In one group of eight birds laying was stopped by the insertion of a thread in the magnum. When the thread was removed, seven of the eight birds began to lay normally within two weeks after the operation.

A second group of eight birds, which had a thread in the magnum was allowed to go through the usual non-laying period following the operation. As in earlier experiments these hens began to lay 30 to 40 days after the operation. As they returned into production a second operation was performed, the original thread was removed and a new one placed in the same general region of the magnum. Six of the eight hens subjected to this treatment continued to lay indicating that the oviduct as a whole loses its sensitivity to respond to the stimulus from the thread.

DISCUSSION

On the basis of these experiments it is suggested that a neural link exists between the oviduct and the pituitary gland of chickens. This is supported by the fact that the presence of an irritant in the magnum suppresses ovulation without affecting the other endocrine functions of the pituitary, the ovary or the responses of their respective target organs. It is known that comb growth in the hen is caused by androgen and that androgen secretion in turn is caused by LH stimulation of the interstitial cells of the ovary (Nalbandov, unpublished). Since in hens in which a thread was present in the magnum, comb-size remains unaffected (indicating continued secretion of *some* LH), the assumption becomes necessary that in the chicken ovulation is caused by the secretion of additional amounts of LH over and above that quantity of LH which is assumed to be produced at a constant rate. It is thought that the occurrence of these "ovulatory peaks" of LH are specifically prevented by the presence of an irritant in the magnum. This interpretation is supported by the observation that the injection of exogenous LH or of progesterone which releases endogenous LH from the pituitary, causes ovulation in hens with thread in the magnum, which stops as soon as injections are discontinued. This interpretation fits in with findings of Sawyer, Everett and Markee (1948, 1949) who showed that in the rat neurogenic activation of the pituitary is necessary for the release of LH. In the hen in which a thread is placed in the oviduct the neural pathway to the pituitary appears to be blocked.

It is of interest to note that in operated hens follicles which normally would have ovulated within a few hours, remained in a state of "suspended animation" over prolonged periods of time, in some cases for more than four weeks, without becoming atretic. Such follicles continued to secrete estrogen since the oviducts were maintained in size and retained their ability to secrete albumen. Furthermore, the follicles remained capable of ovulating over prolonged periods of time, as shown by the fact that ovulations resulted following injection of exogenous LH.

The interpretation presented fits most of the facts observed. There are certain findings which cannot be fitted into this scheme at the present time and which will require additional experimentation. Thus in some of the operated hens, particularly in the early experiments in which key-chains and other large irritants were used, the ovary enlarged considerably, in some cases containing six or more follicles of ovulatory size (30 or more mm. in diameter). This was interpreted to mean that the rate of secretion of FSH and possibily LH was increased appreciably by the presence of the irritant but here too ovulation was suppressed. In some hens the presence of a thread in the magnum did not suppress ovulation but on the contrary caused superovulation. Thus in one case four eggs were laid in two successive days although all of them were soft-shelled. In many other cases two soft-shelled eggs were laid in one day.

The presence of a uterine irritant produces a very high proportion of either soft-shelled eggs or yolks coated only with albumen or even yolks which are laid completely uncoated. In one experiment eight normal hens laid 63 eggs, only one of which was soft-shelled while eight operated hens ovulated with LH produced 16 eggs, eight of which were either soft-shelled or consisted of yolks coated with albumen only. One of these hens laid two eggs in 12 hours, the first egg with a normal shell, the second soft-shelled.

These facts show that uterine irritation may occasionally cause an increased rate of both FSH and LH secretion leading to superovulation.

The existence of this hitherto unsuspected neurogenic connection between the oviduct and the pituitary gland raises the question whether this relationship has any physiological significance in the normal hen or whether it is an artifact. It is thought that the mechanism described is physiologically important in that it may serve the hen as a timing device which prevents ovulation while a yolk (an irritant) is passing through the oviduct. Additional work will be needed before the details of such a signalling system are understood and can be fitted into the time schedule already worked out with regard to the release of LH and the length of time an egg normally spends in the oviduct and in the shell gland.

SUMMARY

The presence of an irritant, such as a loop of thread, in the magnum portion of the oviduct of laying hens, completely suppresses ovulation in the great majority of the treated animals. There is no reduction in the size of the ovary, the oviduct or the comb of these hens for as long as 25 days.

Injection of progesterone or of gonadotrophin containing LH into such hens causes ovulations but only so long as the injections are continued.

The presence of an irritant is thought to prevent the secretion of amounts of LH sufficient to cause ovulation (ovulatory peaks), but does not interfere with the secretion of levels of LH sufficient to assure normal androgen secretion, as is evidenced by the fact that in hens with a thread in the oviduct the comb does not decrease in size. The presence of the loop does not prevent the secretion of normal amounts of FSH since the ovary remains of normal size for as long as 25 days after the operation. For approximately the same length of time the ovary also contains follicles of ovulatory size (30 mm. or over) and in the same numbers as would be expected in unoperated hens. These follicles continue to secrete estrogen at normal levels since the oviduct in operated hens does not decrease in size and remains capable of secreting albumen.

This neurogenic system may be of physiological importance in the normal hen in that it may serve to synchronize ovulation with the other events of the laying cycle.

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THE POTENCY OF CERTAIN DESOXYCORTICOSTERONE ESTERS WITH RESPECT TO THE PRODUCTION OF HYPERTENSION AND CARDIOVASCULAR LESIONS*

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OF THE 11-desoxycorticoids which are available for therapeutic use, desoxycorticosterone acetate (DC-acetate) has enjoyed the widest acceptance. In an effort to increase the activity of the hormone either by conferring upon the steroid a more favorable absorption rate or reducing the rate at which it is metabolized by the body, various esters, in addition to the commonly used acetate, have been prepared. The water soluble DC-glucoside has proven to be very effective, when administered intravenously, in effecting the recovery of adrenalectomized dogs from the symptoms of acute adrenal insufficiency (Meier *et al.*, 1944; Swingle *et al.*, 1952). DC-benzoate, DC-palmitate and DC-butyrate have each been found, when given in identical dosage by single injections, to prolong the life of adrenalectomized rats more effectively than either dexoxycorticosterone or its acetate.

With the recent preparation of DC-trimethylacetate and DC-phenylacetate, attention has again been focused on the metabolic effects of desoxycorticosterone esters. Both of the latter compounds have been found to be more effective than the acetate when given as single injections, in maintaining the adrenalectomized dog free of insufficiency symptoms (Swingle *et al.*, 1952; Gross and Tschopp, 1952), or in prolonging the survival period of adrenalectomized rats (Gaunt *et al.*, 1952; Gross and Tschopp, 1952). Clinically, the trimethylacetate has been found, again administered as a single injection, to have prolonged effects in patients with Addison's disease and acute effects of overdosage have not been observed (Frawley and Forsham, 1951).

There is no reason *a priori* to suppose that the different esters of desoxycorticosterone would vary qualitatively in their actions, although quantitatively dissimilar activities might be anticipated. In view of the fact that toxic symptoms of overdosage with DC-acetate, including hypertension, edema and cardiac failure have been observed in man (Giraud

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et al., 1950; Willson *et al.*, 1940; Thorn *et al.*, 1940) and the rat (Selye and Hall, 1944; Friedman *et al.*, 1948), it might be expected that other desoxycorticosterone esters would display similar effects. Because the studies on DC-phenylacetate and DC-trimethylacetate reported to date appear to have been concerned exclusively with the effects of a single injection, an experiment designed to test the effect of chronic treatment with desoxycorticosterone esters in order to assess their relative toxicity with respect to the cardiovascular system appeared to be warranted.

METHODS

Forty female albino rats of the Holtzman strain weighing between 60 and 89 gms. with an average of 75 gms., were uninephrectomized and divided into four equal groups. The rats of groups 1, 2, and 3, each received 2 mg./day of DC-acetate,† DC-phenylacetate and DC-trimethylacetate respectively, by subcutaneous injection, in 0.2 cc. of the suspension medium. The animals of group 4 each received 0.2 cc./day of suspension medium and served as controls. All animals were maintained on a regimen of Purina Laboratory Chow and 0.9% NaCl solution *ad lib.*

Blood pressures, on unanaesthetized animals, were measured on the first day and once weekly thereafter until the experiment was terminated. A modification (Sobin, 1946) of the tail plethysmograph method (Williams *et al.*, 1939) was employed, and the arithmetic mean of four consecutive pressures agreeing to within 5 mm. Hg was taken to be representative of the blood pressure of the rat in question. The daily fluid intake of each of the groups of experimental animals was measured. The animals were sacrificed on the 28th day of the experiment, and the various tissues and organs were excised and placed in Bouin's solution for fixation and subsequent histologic treatment. After fixation, the organs to be weighed were transferred to water for 24 hours. They were then carefully dissected free of connective tissue, blotted on filter paper and weighed on a torsion balance.

RESULTS

Fluid Intake

The fluid intake of each of the four groups is given in Figure 1. It is apparent that the daily consumption of fluid was greatly augmented in each of the steroid treated groups, although those receiving DC-acetate showed the greatest evidence of polydipsia, and those receiving DC-trimethylacetate the least. Until the third week of treatment, at which time hypertension became more marked in the DC-trimethylacetate group, these animals were consuming about twice as much of the saline solution as controls, while those receiving DC-acetate consumed about three times as much. The fluid intake of rats receiving DC-phenylacetate, while slightly less than that of the DC-acetate group, paralleled the latter closely throughout the major part of the experiment.

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† The authors are indebted to Dr. Robert Gaunt, Ciba Pharmaceutical Products Inc., Summit, N. J. for the desoxycorticosterone phenylacetate and trimethylacetate used in these studies, and to the Schering Corporation, Bloomfield, N. J. for the desoxycorticosterone acetate.

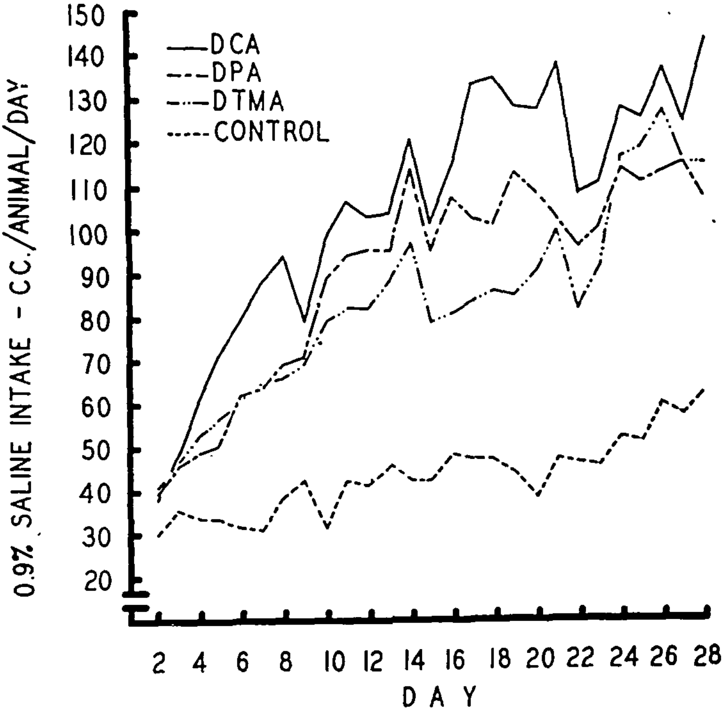

Fig. 1. Intake of 0.9% NaCl solution in control rats and rats receiving the acetate, phenylacetate and trimethylacetate esters of desoxycorticosterone.

Blood Pressure

In the various studies on hypertension which we have conducted in the rat, rarely is a pressure of 135 mm. Hg or higher encountered in unanesthetized normal animals from our colony. However, since an occasional rat may show pressures of 145 mm. Hg, a level of 150 mm. Hg has arbitrarily been selected as the lowest pressure consistent with a diagnosis of hypertension. While it is possible that a few animals having pressures which lie between 140 and 150 mm. Hg might be truly hypertensive while not considered as such, at least the level of 150 mm. Hg permits us to be more certain that such a pressure has resulted only through treatment.

No evidence of hypertension was encountered at any time in the control animals of group 4. The behavior of groups 1 and 2, receiving the acetate and phenylacetate respectively was essentially similar. There was early onset of hypertension, detected in the second week of treatment,which became progressively more severe, with the result that on the 26th day of treatment all of the animsls in these groups were hypertensive, the group average being in excess of 210 mm. Hg. The animals of group 3, receiving trimethylacetate, were much less affected. While there was an early onset of hypertension in four of the animals, i.e. on the 7th day, the incidence did not increase until the fourth week of treatment in which, for the first time, the average blood pressure of the group exceeded 150 mm. Hg. At the last blood pressure determination, the group average was less than 190 mm. Hg and two of the nine survivors were normotensive, having pressures below 140 mm. Hg. Hypertension in group 3 tended therefore, to be later in onset, milder in severity and of lesser incidence than in groups 1 and 2. Table 1 summarizes the data.

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ESTERS ON BLOOD PRESSURE OF THE RAT

Day	Blood pressure	Controls (group 4)	Desoxycorticosterone		
			Acetate (group 1)	Phenyl-acetate (group 2)	Trimethyl-acetate (group 3)
1	No. hypertensive Group average, mm. Hg.	0 112 ±2†	0 112 ±2	0 111 ±2	0 115 ±3
7	No. hypertensive Group average, mm. Hg.	0 111 ±2	4 142 ±6	1 135 ±4	4 148 ±4
14	No. hypertensive Group average, mm. Hg.	0 115 ±3	5 158 ±11	7 156 ±4	3 143 ±6
21	No. hypertensive Group average, mm. Hg.	0 115 ±2	9 176 ±11	10 193 ±7	4 147 ±10
26*	No. hypertensive Group average, mm. Hg.	0 119 ±2	9 218 ±11	9 214 ±10	7 183 ±12

* Nine animals surviving in each steroid treated group on this day.
† Mean ±S.E.

Miscellaneous Observations

The animals of groups 1 and 2, which showed early onset of severe hypertension and marked polydipsia also developed edema and ascites during the second week of treatment and continued to manifest these changes in varying degree throughout the period of treatment. The animals of group 3, treated with DC-trimethylacetate, increased their fluid intake markedly, i.e. from about 90 cc./rat/day to about 125 cc./rat/day during the last week of treatment, at this time they also began to develop the edema and ascites which had an earlier onset in groups 1 and 2, and an additional three animals became hypertensive.

Retinal hemorrhages, a not uncommon symptom of hypertensive oculopathy in DCA treated rats, was observed in two animals in group 1 and one animal in group 2. They did not occur in groups 3 and 4.

Organ Weights: These data are given in Table 2.

Adrenals. There was no significant alteration in adrenal weight as a result of treatment with the DC-esters. This is in conformity with published observations on the effect of DC-acetate. Since the main effect of 11-desoxycorticoids is to cause involution of the cortical zona glomerulosa, which constitutes an insignificant proportion of the total bulk of the gland, changes in adrenal weight are never pronounced in animals treated with these hormones.

Kidneys. The renal enlargement, so characteristic of DCA treated rats, was equally marked in rats treated with DC-phenylacetate and DC-trimethylacetate; there being no significant difference in renal weight between any of the steroid treated groups.

TABLE 2. ORGAN WEIGHTS AND CARDIOVASCULAR LESIONS IN CONTROL AND STEROID TREATED RATS

Item	Controls	Desoxycorticosterone		
		Acetate	Phenylacetate	Trimethyl-acetate
Body wt. g. Initial Final	 73 ±3 146 ±17	 75 ±3 141 ±5	 75 ±4 122 ±6	 75 ±3 141 ±4
Adrenal mg./100 g.	24.6 ±1.5	22.0 ±0.9	29.0 ±2.0	23.7 ±1.4
Heart mg./100 g. Incidence and severity of lesions	298 ±14 0 (10)	449 ±43 + (3)* ++ (1) +++ (2) 0 (3)	453 ± 29 + (2) ++ (3) +++ (3) 0 (1)	378 ±27 + (4) ++ (1) +++ (0) 0 (4)
Kidney mg./100 g. Incidence and severity of lesions	621 ±15 0 (10)	1307 ±89 + (3) ++ (4) +++ (2) 0 (0)	1360 ±96 + (2) ++ (3) +++ (4) 0 (0)	1118 ±104 + (2) ++ (3) +++ (2) 0 (2)
Pancreas Incidence and severity of lesions	 0 (10)	+ (5) ++ (1) +++ (0) 0 (3)	+ (3) ++ (2) +++ (3) 0 (1)	+ (0) ++ (3) +++ (0) 0 (6)

* Severity, designated by pluses, is followed by bracketed figure indicating incidence.

Heart. Cardiac hypertrophy was evident in each of the steroid treated groups, although not as marked in the group which received DC-trimethylacetate as in those treated with the acetate or phenylacetate.

Cardiovascular Lesions

Histologic examination of the kidney, heart and pancreas, the latter having proven to be a site of predilection for the necrotizing arteriolitis which occurs in hypertensive DCA treated rats, revealed that typical lesions were present in animals in each of the groups. However they were of lower incidence and lesser severity in the trimethylacetate than in the acetate or phenylacetate treated rats. A summary given in Table 2 synopsizes the data. The severity of lesions are graded on a scale of 0 to +++. In the pancreas the lesions were entirely vascular, of the periarteritic type; in the heart they consisted of either arteriolar necrosis with an associated perivascular accumulation of inflammatory cells, resembling the "Aschoff nodule"; or as an interstitial myocarditis. The renal lesions were those of arteriolosclerosis and necrosis, glomerulosclerosis and occasionally focal infiltrations of inflammatory cells. The incidence of lesions in each of these tissues was approximately equal in the acetate and phenylacetate groups and less in the trimethylacetate treated rats.

DISCUSSION

Insofar as polydipsia and the development of hypertension may serve as criteria in the estimation of mineralocorticoid toxicity, these experiments suggest that DC-acetate and DC-phenylacetate are of approximately equal potency whereas DC-trimethylacetate is somewhat weaker. The conclusion is also supported by the incidence and severity of cardiovascular lesions. Although, it would be hazardous to draw sweeping conclusions from one experiment involving so few animals, in view of the variability of such responses (Rosenberg *et al.*, 1952), the present experiment suggests that DC-phenylacetate is somewhat more toxic than DC-acetate.

The effects of DC-trimethylacetate, as compared with those of the acetate and phenylacetate derivatives, were relatively delayed in onset. This was manifest in saline intake and the development of hypertension. Since we have never observed lesions of the type described in desoxycorticosterone-treated rats unless hypertension was present, and since the severity of the lesions is correlated with the duration and severity of hypertension, it was not surprising that the cardiovascular lesions were of lower incidence and lesser severity in the DC-trimethylacetate group.

The conclusion which appears to be best compatible with the results obtained is that each of the esters is capable of producing hypertension and cardiovascular damage, but that DC-trimethylacetate, possibily because of a slower absorption rate, requires a longer period of time to exhibit the same toxic potentiality as the acetate and phenylacetate.

Since the evidence previously cited indicates that at a given dosage DC-trimethylacetate exerts a more prolonged effect than the acetate, and inasmuch as it is more effective than DCA even at a lower dosage in supporting life and growth in the adrenalectomized rat (Gaunt *et al.*, 1952), it would seem likely that DC-trimethylacetate will prove, because of its lower toxicity, to be preferable to the acetate in controlling the electrolyte imbalance in patients.

SUMMARY

A comparison of the toxicity of three desoxycorticosterone esters, namely the acetate, phenylacetate and trimethylacetate, has been made in the rat. The criteria used were the induction of hypertension and cardiovascular lesions in the uninephrectomized rat maintained on a high NaCl intake and receiving 2 mg./day of the steroid subcutaneously.

The results indicate that the onset of hypertension, severe polydipsia, edema and ascites is rapid in rats receiving either the acetate or phenylacetate and delayed in rats treated with the trimethylacetate ester.

Cardiovascular lesions, while present in all groups were of lowest incidence and least severity in rats treated with DC-trimethylacetate.

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A QUANTITATIVE STUDY OF THE VASCULARITY OF THE ADRENAL GLAND OF THE RAT DURING PREGNANCY AND AFTER CORTISONE TREATMENT[1,2]

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THE maintenance and activity of cells of an organ is dependent among other things on the functional adequacy of the blood supply. The functional adequacy of the circulation of any organ depends in turn on a number of factors: the blood volume, rate of blood flow, the concentration of gases, nutrients and metabolites in the blood and tissue fluid, the tissue permeability of these substances, and the area of the capillary bed. In an endocrine organ, like the adrenal cortex, these factors are especially important, as the functional activity is expressed by the pattern of blood circulation. The complexities in the morphology of the adrenal cortex of mammals are so great that it seems practical at the present time to approach experimentally only the estimation of the area of the capillary bed. This area may be expressed as a ratio of the surface of the capillary bed to the volume of tissue occupied by it (S. V.).

There are no quantitative estimations of the vascularity in the adrenal gland despite several good qualitative approximations (Sjöstrand, 1934; Gersh and Grollman, 1941; Bennett and Kilham, 1940). The difficulties in the way of a quantitative estimation are apparent. It was hoped that many of these difficulties, if not most, could be avoided by the use of fixation by freezing and drying. Such a study has been made by Gersh and Still (1945) for fat tissue, and essentially the same technique was used in this study.

The experiments were undertaken in normal female and pregnant rats, with and without cortisone treatment. After very rapid fixation of the adrenals in situ by freezing with flowing isopentane at about −150° C., blocks of tissue were removed and dried at a low temperature. They were

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[2] We acknowledge the participation of Dr. Isidore Gersh from the Department of Anatomy, The University of Chicago in preparing this study.

embedded in celloidin and sectioned serially. Camera lucida drawings of the outlines of the capillaries containing red blood cells were made. From the drawings were obtained data that gave the ratio of the total capillary surface to the volume of tissue supplied by the functioning vessels (S/V ratio), and the mean diameters of the capillaries.

It was found that marked changes of the S/V ratio and of the mean diameter of the capillaries occurred in the zona fasciculata and in the zona reticularis during pregnancy and after cortisone acetate treatment. In marked contrast is the relative constancy of the vessels in the zona glomerulosa. The changes in the medullary capillaries, if significant, were small.

MATERIAL AND METHODS

Preparatory phase. Twelve female rats of the Sprague-Dawley strain were used. They were fed "Complete Rockland Rat Diet" and they were allowed water *ad libitum.* Three normal rats served as controls; 3 non-pregnant rats were treated with 3 mg. cortisone acetate daily for 19 days. Six animals in proestrus were caged with males in the afternoon and when sperm were identified in the vaginal smear on the following morning this was considered as day one of pregnancy. Three of these rats were treated with 3 mg. cortisone acetate daily for 18 days; the other 3 received no treatment. Table 1 presents the summary of the weights of the animals at the beginning and at the end of the preparatory phase.

TABLE 1

No.		Weight at the beginning, gms.	Weight after 18–19 days, gms.
1		192	221
2	Controls	196	219
3		188	217
4	Non-pregnant rats. Daily 3 mg.	183	148
5	Cortisone acetate	196	165
6		182	156
7		187	280
8	Pregnant rats	185	269
9		188	267
10	Pregnant rats 3 mg. Cortisone	193	240
11	acetate daily	196	251
12		201	251

Summary of the weights of the animals at the beginning and at the end of the preparatory phase.

Preparation of the adrenals. Under light ether anesthesia the abdominal cavity was rapidly opened, the left adrenal exposed and immediately frozen in situ by a stream of isopentane chilled to about −150° C. The region including the frozen adrenal was then broken out with a chilled bone forceps. The gland was transferred to the drying chamber, and dried at −25° C. for eight days (Gersh, 1932). The adrenals were then placed in absolute alcohol at −25° C., and after 24 hours allowed to warm up to room temperature.

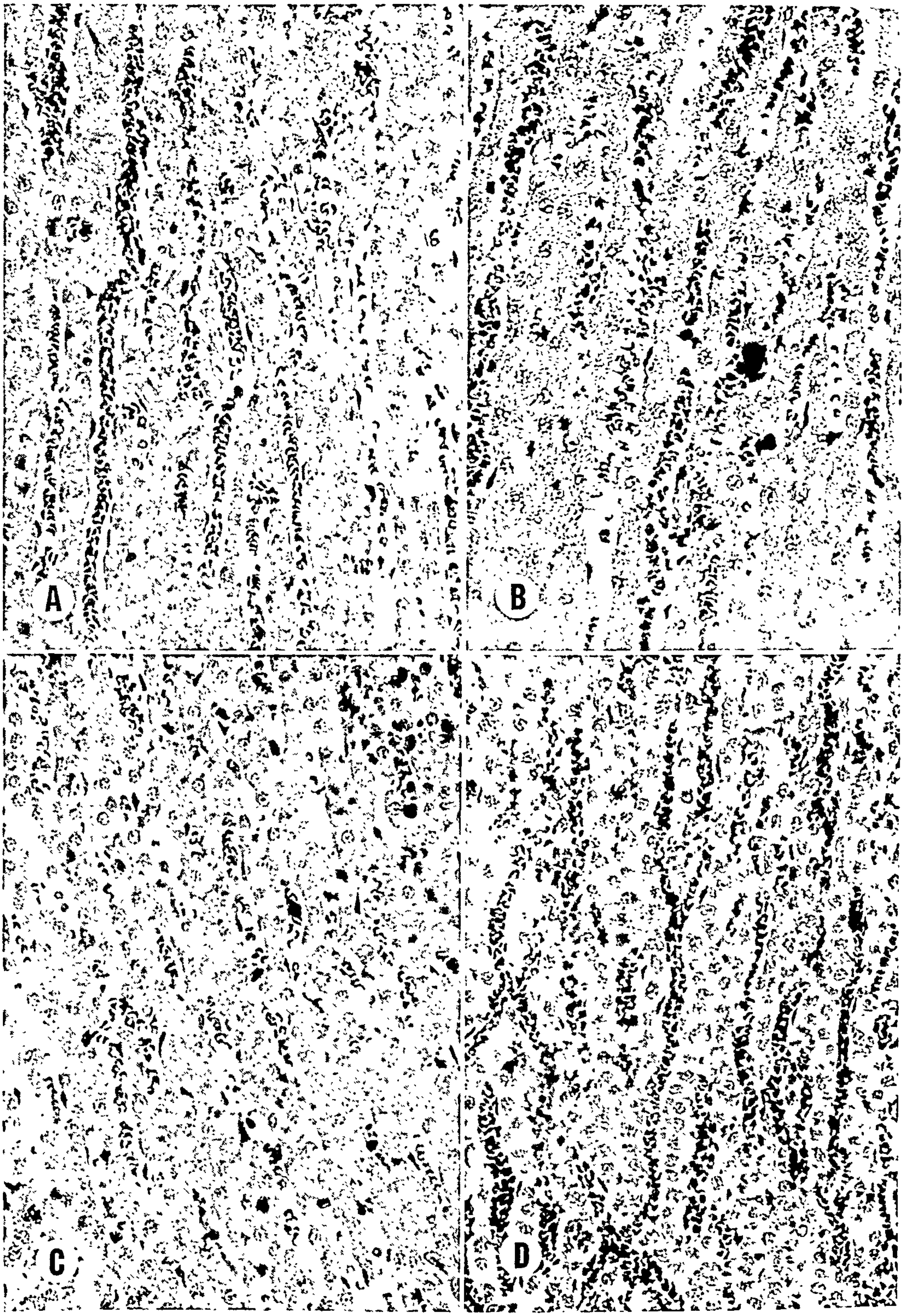

Plate I

TABLE 3

		Controls	3 mg. cortisone acetate daily	Pregnant rats	Pregnant rats 3 mg. cortisone acetate daily
Zona glomerulosa	Width	68.7	90.0	64.2	107.3
	Volume mm.^{3}	3.4	2.8	3.3	3.1
	Total capillary surface mm.^{2}	1176	1056	1003	1062
Zona fasciculata	Width	833.6	529.0	817.0	585.0
	Volume mm.^{3}	25.8	9.1	24.6	10.4
	Total capillary surface mm.^{2}	20744	3365	31857	2552
Zona reticularis	Width	401.8	242.7	370.5	283.5
	Volume mm.^{3}	4.5	1.8	3.9	2.0
	Total capillary surface mm.^{2}	5115	1523	5846	1168
Medulla	Width	703.3	656.9	737.5	597.5
	Volume mm.^{3}	1.6	1.2	2.2	0.9

The width, volume and total capillary surface of the different zones under the different experimental conditions. Marked changes in the total capillary surface in the zona fasciculata and zona reticularis are in contrast to the relative constancy of the vessels in the zona glomerulosa. The total surface of functioning capillaries of the medulla was not determined because measurements of the medullary sinusoids and veins were not made.

the pregnant rats the S/V ratio was significantly lowered and the mean diameter of the capillaries was reduced below that of untreated controls (3.6 microns for both treated groups) (Fig. 2 A, B, C, D.).

These differences in the surface area of capillaries per unit volume are magnified when one takes into account changes in the volume of the fascicular zone (see Table 3). The total capillary surface of the zone showed an increase from 20,747 mm.^{2} to 31,857 mm.^{2} in pregnancy and a decrease to 3,365 mm.^{2} and 2,552 mm.^{2}, respectively after cortisone treatment.

Zona reticularis. There was a definite increase of the S/V ratio during pregnancy and possibly an increase of the mean diameter of the capillaries

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EXPLANATION OF PLATE I

FIG. 2. Photomicrographs to illustrate changes in the diameter and in the number of open capillaries of the zona fasciculata of the rat are more numerous and their lumens are wider in diameter than in the non-pregnant rat. After treatment with cortisone the diameter and the number of open capillaries were markedly reduced in both pregnant and non-pregnant animals. The adrenals were prepared by freezing and drying, embedding in celoidin, and staining with hematoxylin eosin (×550).

A. Normal, non-pregnant. B. Normal, pregnant. C. Non-pregnant, treated with cortisone, and D, pregnant, treated with cortisone.

(from 9.3 microns for normals to 10.0 microns for pregnant animals). (See Table 2.) After cortisone treatment of non-pregnant and pregnant rats the S/V ratios were reduced below figures obtained in the untreated controls. The mean diameter in the non-pregnant group was reduced to 6.4 microns and in the pregnant group to 6.7 microns after cortisone administration.

The total capillary surface in the zona reticularis was increased during pregnancy (5,115 mm.^{2} for normal rats, and 5,846 mm.^{2} for pregnant rats). Cortisone acetate treatment caused a definite decrease of the total capillary surface of the reticular zone in non-pregnant rats (1,523 mm.^{2}) as well as in pregnant animals (1,168 mm.^{2}).

Medulla. A slight decrease of the S/V ratio which may be not significant was observed after cortisone treatment. The mean diameter of the capillaries was reduced from 8.5 microns (non-pregnant) and 7.5 microns (pregnant) to 6.2 microns and 6.0 microns, respectively; whether these changes are significant is also not known. No changes occurred in the pregnant rats. The total capillary surface was not determined in the medulla because measurements of the medullary sinusoids and veins were not taken. As these constitute a significant proportion of the total medullary volume the values of the total capillary surface in the absolute volume of the medulla would be misleading.

DISCUSSION

The very rapid fixation of the adrenals in situ by freezing at about −150° C. makes it possible to determine approximately the dimensions of the capillaries containing red blood cells at the moment of freezing (Gersh and Still, 1945). The knowledge of the so obtained S/V ratio of the different zones of the adrenal contributes to the understanding of the functional activity of the gland.

In contrast to findings in other species several investigators (Donaldson, 1924, 1928; Anderson and Kennedy, 1933; Blumenfeld, 1934) did not find a significant increase in adrenal weight during preganncy. However, a definite increase of the S/V ratio and of the total capillary bed of the zona fasciculata and zona reticularis was observed during pregnancy in these experiments. The S/V ratio for the zona reticularis of the pregnant rats (1,483) is the highest S/V value ever reported for tissue. Catchpole and Gersh (1947) calculated for the medulla of the mouse kidney 1,400, for the kidney cortex 850, for the heart muscle of man 1,184, for the mouse liver, 800. The values for other tissues (skeletal muscle, brain cortex, cerebellum, duodenum, pancreas) range between 190 and 500.

Severe adrenal atrophy in rats has been described after high dosages of cortisone (Ingle and Mason, 1938; Wells and Kendall, 1940; Stebbins, 1950; Winter *et al.*, 1950) due to reduction in size of the cells of the fascicular and reticular zones. A complete loss of lipid from the cells of these zones has been observed. Sourkes and Henage (1951) stated that adrenals of cortisone treated rats showed a decreased endogenous respiration as well as greatly reduced rates of oxidation of various intermediates of the Krebs' cycle. While the total volumes of the zona fasciculata and zona reticularis were reduced to nearly one third of the original volumes after cortisone acetate treatment, the total capillary surface of these zones was reduced to approximately one fifth to one tenth of the capillary bed of the controls and of the pregnant adrenals.

Sjöstrand (1934) stated that the same factors which are responsible for excessive cortical activity are effective on the vascular pattern of the medulla. Gersh and Grollman (1941) reported that the same vascular adjustments appear to take place in the medulla during hyperactivity as were described in the cortex, though to a less marked degree. It seems that no change takes place in the medulla of the adrenals of pregnant rats. The decrease in the S/V ratio and in the mean diameter of the capillaries was so small after cortisone treatment that these changes may be considered also as not significant.

SUMMARY

The ratio of the surface of the capillaries to the volume of tissue supplied by vessels (S/V ratio) of the zona fasciculata was markedly increased in pregnancy, and reduced below normal levels after the daily administration of 3 mg. cortisone acetate for a period of 18–19 days. Similar changes of the S/V ratio were obtained in the zona reticularis.

These differences in the surface area of capillaries became magnified by taking into account the volume of the zones. The total capillary surface of the zona fasciculata was increased during pregnancy by one half. It was reduced by one fifth to one tenth after cortisone acetate treatment.

Changes of the vascularity of the zona glomerulosa were not observed under the different experimental conditions. The S/V ratio in the medulla was not altered markedly in the pregnant animals and the slight decrease of the S/V values after cortisone treatment was too small to be considered significant.

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EFFECTS OF TESTOSTERONE PROPIONATE ON HISTOCHEMICAL REACTIONS OF RAT SEMINAL VESICLES

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INTRODUCTION

THE English anatomist, John Hunter (1786), was the first to describe the seminal vesicles of the rat. His description is as follows: "the bags are large and flat with serrated edges and lie someway within the abdomen, containing a thick ash coloured mucus, nearly the consistency of soft cheese. . . ." Nearly 150 years later Moore, Hughes, and Gallagher (1930) made a detailed cytological study of this organ and demonstrated the fundamental relationship existing between the testicular hormone and the functional activity of the secretory epithelium. Recently Porter and Melampy (1952) have presented the results of a biochemical study of the composition and metabolism of this gland.

This investigation deals with the effects of testosterone propionate on the structure and function of the seminal vesicles of the rat. Histochemical observations were made on basophilia, the localization of alkaline and acid phosphatases, and periodic acid-reactive carbohydrates. Furthermore, information is presented on the distribution of connective tissue in the gland as well as mitotic activity. Intact, castrate, and hormone-treated castrate animals were used in this work.

MATERIALS AND METHODS

Histochemical changes in the seminal vesicles of the rat were studied in 3 groups of animals: (1) 6 intact sexually mature males, (2) 19 castrates, 2, 5, 10 and 20 days after gonadectomy, and (3) 23 castrates receiving subcutaneously 500 μg. of testosterone propionate (T.P.)† daily for 2, 5, 10, and 20 days. The seminal vesicles of the animals in group 3 were allowed to atrophy for a 20-day period following castration prior to hormone treatment. The animals in groups 2 and 3 were mature males weighing 240–255

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† Peranderen, Ciba Pharmaceutical Products, Inc., Summit, N. J.

ble sources of error in the use of purified ribonuclease in cytochemical studies have been discussed by Stowell and Zorzoli (1947).

The lamina propria contained methylene blue-staining cells, pink fibers, and brightly stained erythrocytes and no basophilia was observed in the arterioles and venules. The secreted material present in the lumen was stained with eosin.

Alkaline and acid phosphatases. The results obtained by a histochemical investigation of the influence of male sex hormone on the alkaline and acid phosphatase activity of the seminal vesicles of the rat as determined by the method of Goetsch, Reynolds, and Bunting (1952) are presented in Tables 1 and 2 and Figures 5, 6, 7, and 8. A gradient of apparent alkaline phosphatase reaction occurred adjacent to the interacinar stroma in sections deparaffinized prior to incubation which makes critical evaluation of the localization difficult. Furthermore, the acid phosphatase reaction in deparaffinized sections was somewhat variable. Incubation of non-deparaffinized tissue sections gave a more accurate localization of enzyme activity. No alkaline or acid phosphatase activity was found in air-dried smears of seminal vesicle secretion. These results confirm chemical

TABLE 1. ALKALINE PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	0*	0	0	0	0	0
B. Cytoplasm	0	0	0	0	0	0
C. Secretion granules	0	0	0	0	0	0
Connective tissue (lamina propria)	+++	+++	++	++++	+++	+
Blood vessels in lamina propria	+++	+++	++	++++	+++	+
Smooth muscle	+	0	0	0	0	0
Blood vessels in smooth muscle	+	++	+	+++	+	+

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

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EXPLANATION OF PLATE I

FIG. 1. Basophilia in seminal vesicles of intact animal. Toluidine blue. ×160.

FIG. 2. Same as Fig. 1. Following ribonuclease prior to toluidine blue. ×160.

FIG. 3. Basophilia in seminal vesicles of intact animal. Eosin-methylene blue. ×500.

FIG. 4. Seminal vesicles of 20-day castrate. Atrophied secretory epithelium. Eosin-methylene blue. ×500.

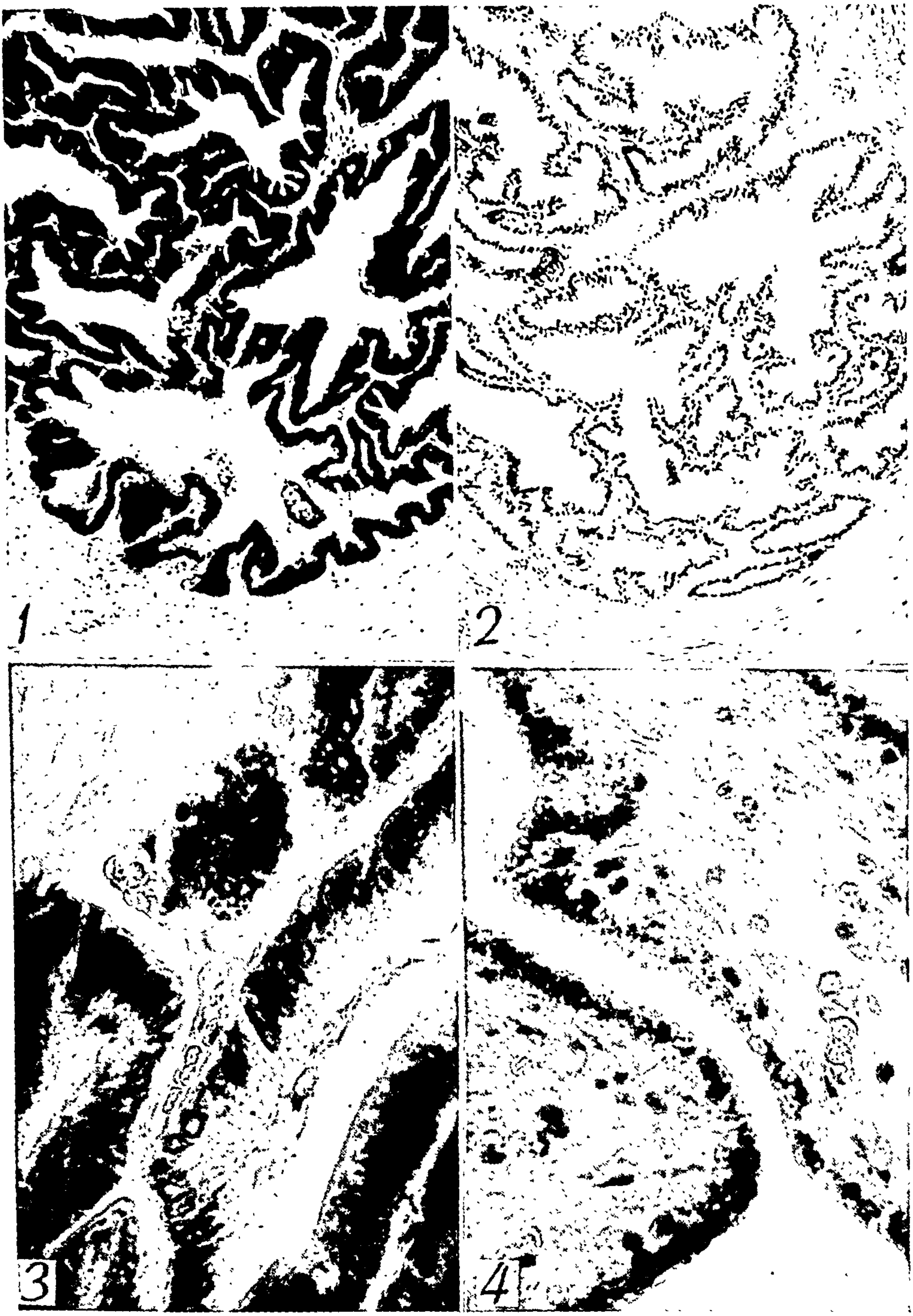

PLATE I

analyses which indicated the absence of both enzymes in the secretion of the rat.

Connective tissue. The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The lamina propria was formed chiefly of elastic fibers, and numerous arterioles and venules were widely distributed throughout this structure. No basement membrane was visible as such and the epithelium was seen to rest directly on the connective tissue as previously described by Moore, Hughes, and Gallagher (1930). The lamina propria divided into secondary and tertiary

TABLE 2. ACID PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	+++*	++	+	+++	+++	+++
B. Cytoplasm	+++	†	†	+++	+++	+++
C. Secretion granules	+++	0	0	++++	++++	++++
Connective tissue (lamina propria)	+++	++	+	++++	++++	++++
Blood vessels in lamina propria	+++	++	+ or ++	++++	++++	++++
Smooth muscle	++	+	+	+++	+++	+++
Blood vessels in smooth muscle	++	++	+ or ++	+++ or ++++	+++	+++

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

† Reaction appeared to decrease but because of the small amount of cytoplasm present in each cell it was difficult to evaluate the intensity of the reaction.

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EXPLANATION OF PLATE II

FIG. 5. Alkaline phosphatase localization in connective tissue of seminal vesicles of intact animal. No reaction in epithelium and secretion in lumen. Two hours' incubation. ×160.

FIG. 6. Alkaline phosphatase in the seminal vesicles of a 5-day T. P. injected castrate. A stronger reaction was obtained in the connective tissue of this stage than in Fig. 5. Two hours' incubation. ×160.

FIG. 7. Seminal vesicle tissue of a 20-day castrate showing acid phosphatase localization. Four hours' incubation. ×160.

FIG. 8. Acid phosphatase in the seminal vesicles of a 5-day T.P. treated castrate. Intense activity at the apical end of the epithelium. Four hours' incubation. ×160.

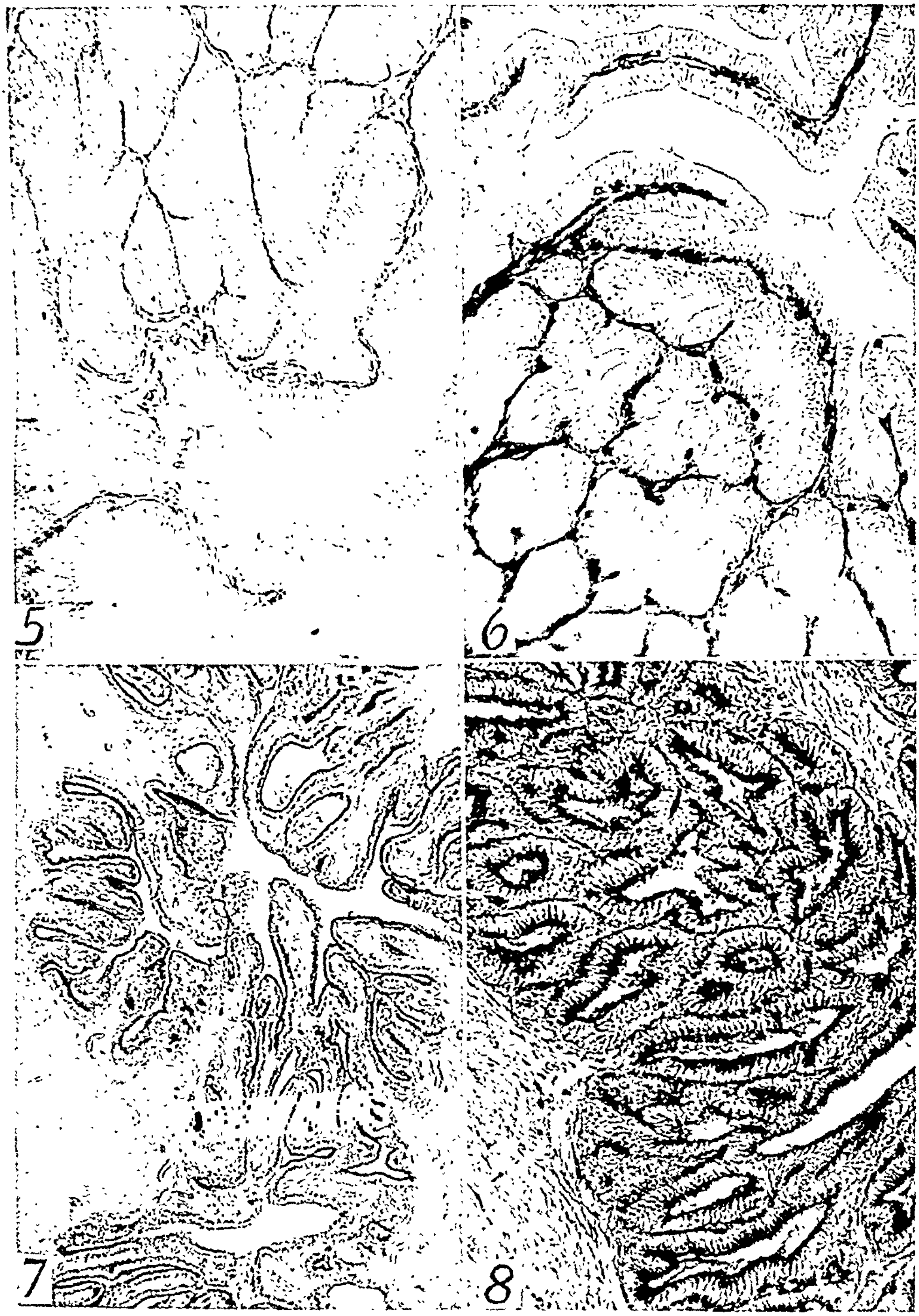

PLATE II

DISCUSSION

In a biochemical investigation of the seminal vesicles of the rat (Porter and Melampy, 1952) it has been demonstrated that protein synthesis is an important function of this organ and is androgen controlled. The secretion produced by this organ in normal males contained 13.6% total nitrogen on a dry-weight basis. Castration of mature rats caused a decrease of 73% in the fresh weight of the secretion-free glands after 20 days and there was a 74% decrease in the total nitrogen content of this organ in the same interval. The catheptic activity decreased to 0.9 of a unit after 24 days in contrast to 2.8 units at the time of gonadectomy. Two days following castration there was a 78% decrease in the quantity of secretion initially present in the glands. The secretion had decreased 98% by the fifth day, 99% by the tenth day and was completely absent by the twentieth day. Subcutaneous injection of testosterone propionate (500 μg.) per day into castrate rats for 20 days resulted in the following increases per gland: wet weight 48 to 525 mg., nitrogen 0.2 to 7.3 mg. and the amount of secretion 0 to 1322 mg. The catheptic activity per gland of rats that were injected over a 10-day period increased from 0.9 to 4.5 units during this time.

In the present study it has been possible to extend these observations on the metabolism of the seminal vesicles by the application of histochemical methods for basophilia using toluidine blue and eosin-methylene blue. The presence of ribonucleoprotein in the secretory epithelium of this organ is suggested because the cytoplasmic and in many cases nucleolar basophilic reactions are abolished by treating sections with ribonuclease. This basophilia indicates an accumulation of ribonucleoprotein which may be related to the endocellular protein-synthesizing mechanism which is functional in this organ of the sexually mature rat. It is of interest that Wislocki (1949) has reported that cytoplasmic basophilia was absent from the epithelium of deer seminal vesicles but the cytoplasm had a strong affinity for acid dyes.

Recent investigations have shown a correlation between the nucleic acid content of cells of various tissues and their activity in protein synthesis. For example, in the pancreas, Caspersson (1950) has demonstrated that there is an intense protein metabolism and that the nucleotides of the nucleoli and cytoplasm are conspicuous. After pilocarpine stimulation there is a rapid synthesis of protein by the acinar cells which is preceded by nucleolar growth. Another example of this relationship is found in the gastric mucosa in which the protein-producing cells are characterized by a strong reaction for ribose nucleotides when compared to the parietal cells.

Deane (1946) in a study of the basophilia of hepatic cells of mice has demonstrated that the degree of the reaction is related to the metabolic activities of the organ. For example, *in the livers of mice killed after a* known feeding time, changes in the amount and distribution of basophilic material occurred. Dempsey and Wislocki (1945) have shown that basophilia is a characteristic feature of the cytoplasm of the syncytium and cytotrophoblast of the human placenta. This reaction in the syncytium was most marked in early pregnancy and disappeared during the last months of gestation. Ribonucleoprotein was also reported as a component of the syncytial trophoblast of the cat and the trophoblast of the sow by Wislocki and Dempsey (1946 a, b). In an investigation of the histochemistry of the rodent's placenta Wislocki, Deane, and Dempsey (1946) observed cytoplasmic basophilia in the labyrinth and yolk sac of the mouse, rat, guinea pig, rabbit and hamster. It decreased gradually in the labyrinth but persisted unaltered in the yolk sac until term.

Rabinovitch, Junqueira, and Rothschild (1951) reported that testosterone propionate increased the amount of ribonucleic acid phosphorus of seminal vesicles of castrate rats. These investigators indicated that their findings support the suggestion of Caspersson (1950) dealing with the relation between ribonucleic acid and protein synthesis.

Lesher (1951) has observed that in the case of the salivary gland of Drosophila larvae there appears to be a close correlation between cytoplasmic basophilia and nucleolar size.

In the present study the nuclei in the secretory epithelium of the seminal vesicles of intact animals were large and elliptical and nucleoli were visible in most of them. Two days after gonadectomy a few of the nuclei were pyknotic and contained small nucleoli. Twenty days following castration many nuclei were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. However, in some instances, these structures were similar to those observed in intact animals and this variation may be due to differences in cellular activity. After 20 days of hormone injection, both nuclei and nucleoli of the secretory epithelium resembled those described as characteristic for intact animals. The changes observed in the nucleolus suggest that this structure is of importance in the secretory activity of the epithelium of the seminal vesicles.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of seminal vesicles of intact animals as well as castrates and hormone-treated castrates (Table 1 and Fig. 5). The connective tissue, particularly the lamina propria and its associated blood vessels, gave a strong reaction which became progressively weaker 10 days after castration. However, after 5 days of hormone injection, the reaction of this tissue became intense but after this time this reaction decreased as is shown in Table 1. This apparent decrease in the histochemical reaction may be related in part to the great enlargement of the acini at this time and as a result the stroma became stretched and relatively thin.

Alkaline phosphatase concentration as measured by its activity per gm. dry weight of secretion-free tissue varied during the 20 days of hormone treatment; this activity was greatest by the fifth day when the average wet weight of the gland was 203 mg., and after this time there was a decrease in activity which was associated with the increase in the size of the gland which was 525 mg. after 20 days of injection (Porter and Melampy, 1952). Due to the greater relative increase in the various constituents of the gland, the absolute activity of this enzyme per unit of weight became less from the fifth to the twentieth day of injection. An intense alkaline phosphatase reaction was observed by Dempsey, Greep, and Deane (1949) in the stroma and capillaries immediately underneath the epithelium of the seminal vesicle of the rat. After hypophysectomy the phosphatase activity declined and finally disappeared, but the rate of disappearance varied with different substrates. Similar results followed gonadectomy. Wislocki (1949) observed in the case of October deer alkaline phosphatase at the distal margins of the epithelial cells of seminal vesicle tissue, in the secretion filling the lumen, and in the interacinar stroma. In June deer much less enzyme activity was present.

The absence of this enzyme from the secretory epithelium of the seminal vesicles of the rat is in accordance with the findings of other investigators which indicate the absence or low concentration of alkaline phosphatase activity in the cytoplasm of cells responsible for globular protein secretion such as in salivary glands (Gomori, 1941), pancreas (Kabat and Furth, 1941), gastric mucosa (Jacoby, 1946), and albumen-secreting regions of the fowl oviduct (Bradfield, 1949). However, the occurrence of this enzyme in the interacinar stroma of this gland is in agreement with the results of earlier studies on collagen formation in wounds (Fell and Danielli, 1943) and bones (Bourne, 1942, 1943), rat uterine epithelium (Pritchard, 1947), the vaginal wall of ovariectomized mice treated with estrogen (Jenner, 1947), and silk glands of spiders and caterpillars (Bradfield, 1950). According to Bradfield (1950) the presence of alkaline phosphatase in these various tissues suggests a relationship between this enzyme and fibrillar protein formation. However, in a recent study Bern and Levy (1952) have presented data which cast doubt on the concept of a relationship between alkaline phosphatase activity and the synthesis of fibrous proteins such as keratin, collagen, and myosin.

In the intact animal the nucleus of the epithelium showed a strong reaction for acid phosphatase which became progressively weaker during the 20 days following castration as shown in Table 2. The cytoplasm and secretion granules likewise gave a strong reaction in the intact animal, but, as the cell atrophied following castration, the intensity of the reaction appeared to decrease in the cytoplasm and no secretion granules were present (Fig. 7). However, 5 days after hormone treatment the reaction was strong in the nucleus and cytoplasm and intense in the granules. The connective tissue and smooth muscle as well as the blood vessels in these tissues showed a decrease in acid phosphatase after castration followed by an increased reaction after hormone treatment (Table 2 and Fig. 8).

Porter and Melampy (1952) found that the acid phosphatase activity on a gland basis as determined by chemical analysis showed a 40% decrease 5 days following castration and a 65% reduction after 20 days. Likewise, during the same interval, alkaline phosphatase activity decreased 81%. There was a striking similarity between the decrease in activities of these enzymes and the loss in weight of the gland. The concentration of acid phosphatase increased 964% after 10 days of hormone injection.

Wislocki (1949) observed that acid phosphatase was localized almost entirely in the nuclei of epithelial cells and was found to a lesser degree in the nuclei of the stroma of the seminal vesicles of the deer. In the case of a June deer the acid phosphatase reaction was very much fainter than that found in October deer. Dounce (1950) has stated that acid phosphatase appeared to be generally present in both cytoplasm and nuclei. Wachstein (1944) in an investigation of renal phosphatases suggested that acid phosphatase was probably of importance in cell metabolism. This concept received further support when a marked increase in the enzyme was found in the perikaryon during chromatolysis (Bodian and Mellors, 1945), and Hard and Lassek (1946) have indicated that acid phosphatase may be of importance in maintaining the myelin sheath. The physiological significance of acid phosphatase in the tissues of the seminal vesicles of the rat is unknown but the activity of this enzyme was observed to be influenced either directly or indirectly by male-sex hormone.

SUMMARY

A basophilic reaction was observed in the cytoplasm of the secretory epithelium of the seminal vesicles of the rat following eosin-methylene blue and toluidine blue which was abolished by ribonuclease. The nucleoli were also basophilic but the reaction following ribonuclease was variable.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of the seminal vesicles of intact animals as well as castrate and hormone-treated castrates. The connective tissue, particularly the lamina propria and its blood vessels gave a strong reaction which became progressively weaker 10 days after castration.

Following 5 days hormone treatment the reaction became intense but after this time decreased. The results obtained from this histochemical study of both acid and alkaline phosphatase activity in seminal vesicle tissue agree with the chemical analysis of similar tissues for these enzymes. These enzymes were absent from seminal vesicle secretion. In the intact animal the nuclei of the secretory epithelium showed a strong reaction for acid phosphatase activity which became weaker following castration. Likewise the cytoplasm and secretion granules gave a strong reaction and the intensity appeared to decrease in the cytoplasm as the cells atrophied. However, after 5 days treatment with testosterone propionate (500 μg. per day) the reaction was strong in the nucleus and cytoplasm and intense in the granules. A similar response was also observed for acid phosphatase activity in the connective tissue, blood vessels, and smooth muscle of the gland.

The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The sites of localization of alkaline phosphatase closely paralleled the distribution of the lamina propria in this gland. Following castration there was an atrophy of the secretory epithelium and the connective tissue and muscle layers were thickened in appearance. The administration of testosterone propionate caused a reactivation of the gland.

Glycogen was absent from the seminal vesicle tissue of the rat. A slight positive reaction for periodic acid-reactive carbohydrates was obtained in the cytoplasm of some of the cells of the secretory epithelium, smooth muscle layer, the elastica interna of the blood vessels, and lamina propria.

It was found by the use of the colchicine technique in both intact animals and 20-day hormone treated castrates that 0.3% of the secretory cells showed mitoses. After one day of injection there was 0.2% dividing cells but following 2 days of hormone treatment there was 13.4%. Two days after gonadectomy a few of the nuclei of the secretory cells were pyknotic and contained small nucleoli; whereas 18 days later many were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. After hormone treatment, both structures resembled those of intact animals.

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EFFECT OF IODIDE AND THYROID ON THE PRODUCTION OF TUMORS OF THE THYROID AND PITUITARY BY PROPYLTHIOURACIL[1]

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IN EXPERIMENTS carried out in this laboratory benign and metastasizing adenomatous tumors of the thyroid gland of rats have been found following prolonged administration (over one year) of propylthiouracil. Similar results have been reported previously by other investigators with thiourea (Purves and Griesbach, 1947; Money and Rawson, 1947), and with thiouracil (Dalton, Morris and Dubnik, 1948; Money and Rawson, 1950). The pathogenesis of the tumors is probably related to an increased production of thyrotropic hormone by the anterior pituitary over a prolonged period. The production or release of other hormones may also be affected, with resulting changes in other endocrine glands.

Ferguson and Sellers (1949) found that the concurrent administration of iodide with thiouracil for short periods (2–6 weeks) lessened the goitrogenic effect and resulted in thyroid glands which were 20–30% lighter than those produced by the goitrogen alone. They attributed this effect to the antithyrotropic action of iodide. This suggested that giving iodide with a goitrogen for a long period might affect the development of tumors produced by the goitrogen. Because of the antithyrotropic activity of the thyroid hormone itself, and in an attempt to maintain the metabolic and growth rates normal, in the experiments to be described dried thyroid was also given with propylthiouracil, the goitrogen selected for the investigation. The result of this treatment was an even greater enlargement of the thyroid gland, and in addition the occurrence of multiple adenomas of the anterior lobe of the pituitary.

EXPERIMENTAL

Two hundred and eighty rats of the Wistar strain, of equal sex distribution, bred in the laboratory, were divided into four groups and were given food and water *ad libitum.* Twenty from each group were kept in individual cages in order to follow the daily intake of food, and the remainder were kept in groups of ten in a cage. Each animal was weighed at weekly intervals.

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[2] Holder of a National Research Council of Canada Medical Fellowship, 1951–52.

Group 0 was fed the usual laboratory ration[3] in powdered form.
Group 1, laboratory ration plus propylthiouracil 0.02%.
Group 2, laboratory ration plus propylthiouracil 0.02% plus sodium iodide 0.02%.
Group 3, laboratory ration plus propylthiouracil 0.02% plus dried thyroid[4] 0.02%

The rats were maintained on this regime for 15 months after which time four rats from each group, chosen at random, were sacrificed at weekly intervals. At necropsy, body and organ weights were recorded and tissues were fixed in Bouin's solution or in formol-saline. After sectioning, tissues were stained with haematoxylin and eosin invariably, and in many cases with special stains for connective tissue, fat, ceroid, cytoplasmic granules, mast cells, etc. The pituitaries were stained with acid fuchsin, methyl blue (Wilson 1952) and often with Gomori's aldehyde-fuchsin stain. (Gomori, 1950). During the experiment the oxygen consumption of representative animals was measured at intervals using the method previously referred to by Sellers and You (1950). The level of iodine bound to plasma-protein was estimated (Barker, 1951) prior to sacrifice in representative groups, and the total iodine content of the thyroid (Kendall, 1920) was measured using that part of the gland not required for histological purposes.

Measurement of radio-iodine uptake (I^{131}) was carried out, and radioautographs were prepared following the method of Leblond and Gross (1948).

Measurement of exophthalmos

At necropsy, photographs were taken from vertically above the head of each rat after removal of the skin. The presence of exophthalmos was measured by dividing the distance between the points of maximum curvature on each cornea, by the distance between the malar processes. If the result was greater than 1, exophthalmos was said to be present; if less than 1, there was no exophthalmos. If the ratio was 1, or the angle of the head made measurements inaccurate, the reading was classed as "doubtful." The results are shown in table 4.

RESULTS

In analyzing the data it was found that expressing the results as a median and range presented more information in concise form than did the average and standard deviation. Occasional very large values rendered the latter method unwieldy, and difficult to interpret.

Rats which received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) gained very little weight during the entire experiment. The fur was coarse and sparse. The weight-curve of the group which received propylthiouracil plus thyroid extract (group 3) resembled that of the controls and at the end of the experiment the average weight was only slightly less than the control value (Table 1). The fur was normally sleek in appearance. In all groups the average food intake was consistent with the average gain in weight, the controls, and rats receiving propylthiouracil plus thyroid consuming almost twice the amount eaten by the other groups.

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[3] Fox breeder cubes, Master Feeds, Toronto Elevators Limited, containing 2.6 mg. iodine per 100 gm.

[4] Dried thyroid powder containing 0.3% iodine, Parke-Davis and Company, Walkerville, Ontario.

Thyroid

Weights: (Table 1). The addition of propylthiouracil to the diet caused a significant increase in the weight of the thyroid gland. When propylthiouracil and iodide were administered simultaneously the increase in weight was less than that observed with propylthiouracil alone, but when propylthiouracil and thyroid extract were administered simultaneously (group 3), the thyroid glands were tremendously enlarged to as much as eighty, and on the average to twenty times the weight of the thyroids of the control animals.

Pathology. Gross: The thyroid glands from animals of the three groups which had been fed propylthiouracil were all enlarged to a variable extent. In some cases the enlargement was diffuse, regular and involved both lobes. In others, one or both lobes had multiple small firm nodules or an occasional marble-sized cystic swelling (Fig. 1). The color was also variable, although glands from groups 1 and 3 (propylthiouracil and propylthiouracil plus thyroid) were deeper red and apparently more vascular than glands from group 2 (propylthiouracil plus iodide).

TABLE 1. WEIGHTS

No. of rats	Group	Sex	Body weight in grams			Thyroid weight, mg.		Pituitary weight, mg.		Adrenal weight, mg.	
			Initial av.	Final							
				Median	Range	Median	Range	Median	Range	Median	Range
15	0	M	152	399	340–484	36.0	27.4– 47.4	11.8	9.2– 18.0	37.9	27.2–45.8
14	control	F	144	275	254–324	28.5	20.2– 39.0	14.7	10.8– 35.7	55.6	28.0–78.8
20	1	M	159	191	142–278	97.7	44.6– 395.2	12.2	7.8– 17.0	24.0	18.1–32.1
17	PTU	F	145	175	119–230	79.8	40.2– 568.0	15.0	9.0– 22.4	24.2	18.2–36.6
14	2	M	166	189	148–211	50.1	27.6– 143.4	11.6	8.2– 15.6	24.2	19.2–29.0
12	PTU+I	F	152	158	108–212	48.6	32.0– 94.0	15.3	7.8–500.0	24.8	18.0–34.6
12	3	M	152	346	278–402	517.1	363.8– 674.1	20.9	10.0–164.0	39.9	32.4–54.0
12	PTU+Th	F	144	257	154–302	442.2	150.0–1660.0	28.6	11.6– 60.6	68.8	42.0–89.4

Microscopic: The microscopic appearance varied greatly within and between treated groups. Large areas (comprising most of the gland) showing

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EXPLANATION OF PLATE I

FIG. 1. Thyroid (wt. 1660 mg.) and pituitary (wt. 22.8 mg.) glands. Propylthiouracil plus thyroid group. Thyroid metastases found in lung. Note small nodule on pituitary (upper right, see figs. 11, 13).

FIG. 2. Pituitary gland (wt. 38 mg.) same group. Note nodule attached to anterior lobe (entire left of photograph). See figs. 16, 17.

FIG. 3. Thyroid. Small circumscribed area containing colloid. (Mallory×40.)

FIG. 4. Thyroid. Colloid "lake." Compressed follicular cells form a pseudo-capsule. (H.+E.×40.)

FIG. 5. Thyroid. Follicular cells surrounding well-stained colloid are stratified and hyperchromatic. (H.+E.×560.)

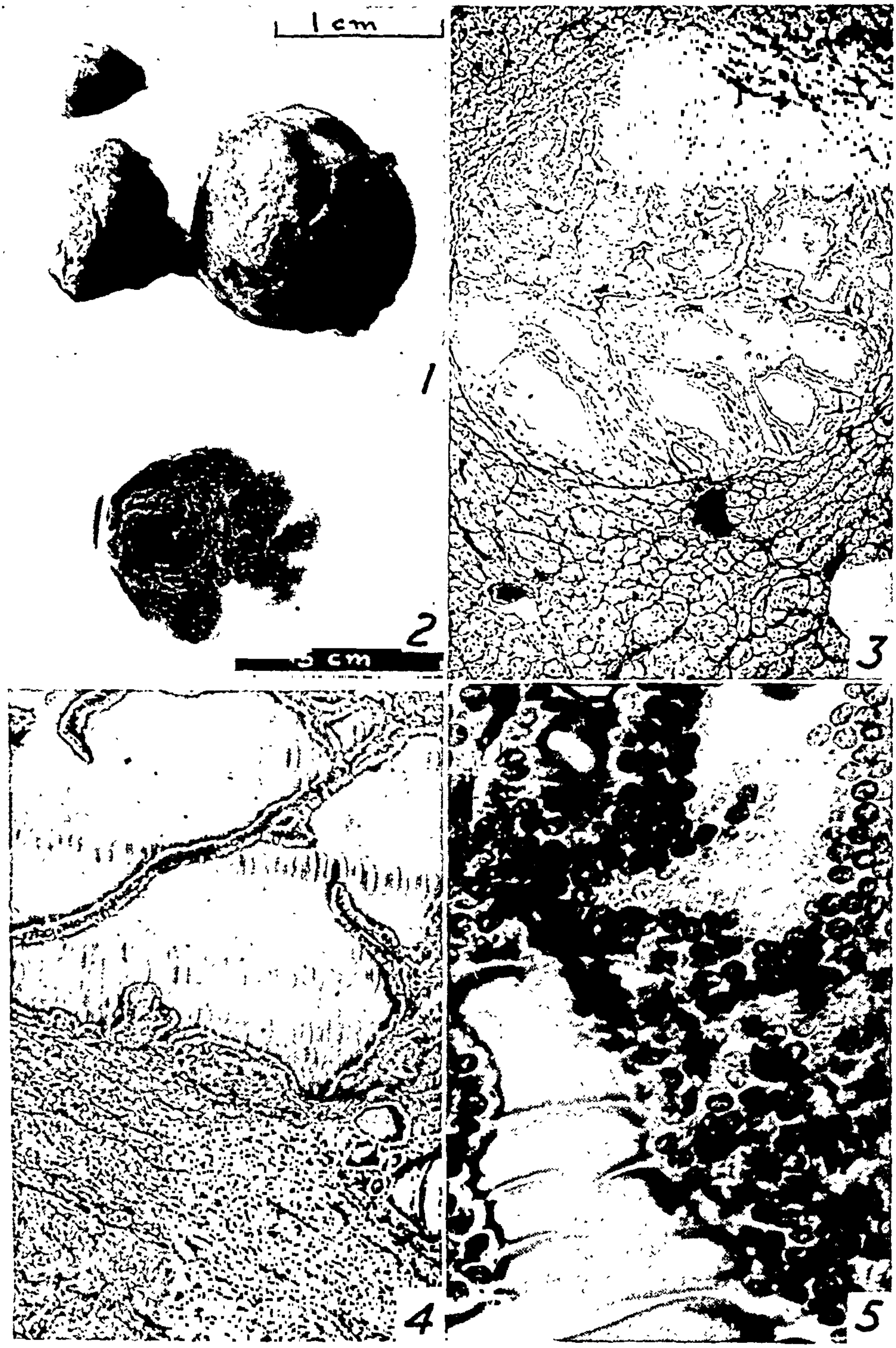

PLATE I

hypertrophy and hyperplasia of the follicular cells, mitotic figures, budding of the follicular wall (with an apparent increase in number of follicles), vacuolation of, or more frequently almost complete disappearance of colloid could be found in every section. Generally these changes were advanced so that the normal architecture of the gland was no longer discernible.

Frequently (in almost every gland weighing more than double the average weight of the normal) an area made up of large follicles containing well stained colloid was demarcated from its surroundings by a pseudocapsule made up of compressed follicular cells or of fibrous trabeculae. The cells within such a nodule showed considerable variation in that sometimes they appeared cuboidal or even flattened, and in other sections, hyperchromatic, columnar cells in layers with many mitotic figures projected and branched into large colloid cysts or "lakes." Wide blood-sinuses, and evidence of hemorrhage into the cysts could be seen. The similarity of some areas to the papillary cystadenomas of human thyroids was a striking feature. Less frequently, circumscribed or semi-circumscribed areas, with little or no colloid and either vacuolated, but hyperchromatic cells, or anaplastic cells were evident (Figs. 3–7).

While the size of the glands (and of gross colloid cysts) was much greater in the group receiving propylthiouracil with thyroid the *presence* of localized colloid areas, of colloid cysts, or of cellular adenomas was not restricted to any one group receiving propylthiouracil. By histological examination it was not possible to assign any tissue-section chosen at random to its correct group.

In 3 or perhaps 4 animals (one—group 1, propylthiouracil; 2 or 3—group 3, propylthiouracil plus thyroid) metastases were found seeded through the lungs. Thyroid follicles containing colloid were found invading the capsule of the gland, in venules in muscle adjacent to the gland, in the

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EXPLANATION OF PLATE II

FIG. 6. Thyroid. Projections from the follicular walls extend into dilated blood sinuses. Note the thin "stem" of the daughter follicle (center). (H.+E. ×150.)

FIG. 7. Thyroid. Hemorrhage has occurred into follicles. In some follicles (upper) necrosed follicular cells, blood pigments and colloid are present. Organization of smaller follicles has taken place. (H.+E. ×40.)

FIG. 8. A follicle of thyroid tissue in a venule adjacent to the gland. Note the intimal layer of the vessel, the red blood cells between it and the follicle, the colloid in the follicle. (Mallory ×900.)

FIG. 9. Thyroid tissue in the submucosa of the esophagus. The epithelium (lower left) and muscular layer (upper) can be seen on either side of the thyroid tissue. (Mallory ×150.)

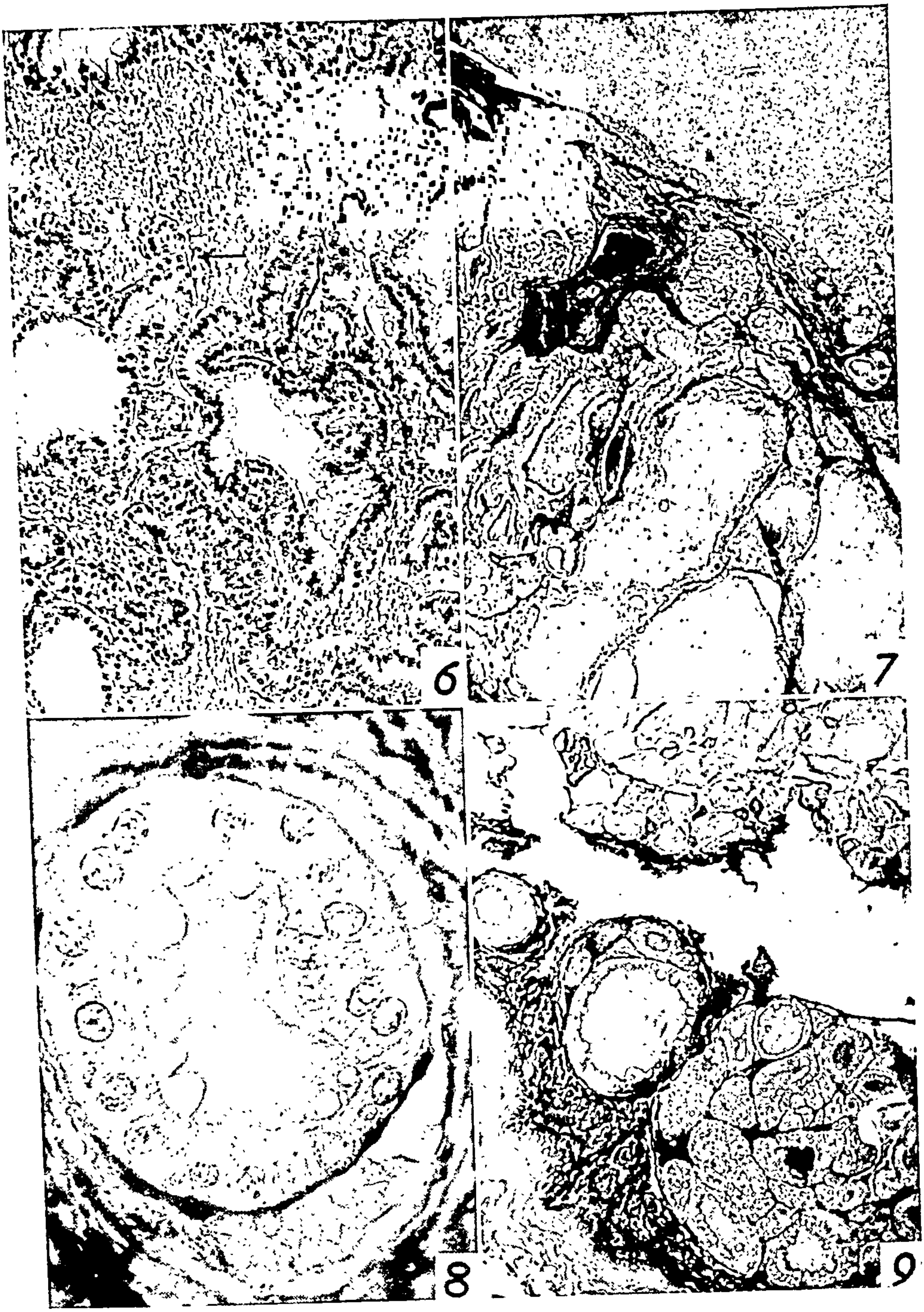

PLATE II

submucosa of the esophagus and in muscle deep to the esophagus, in a branch of the pulmonary artery in the lung and in numerous alveolar vessels (Figs. 8–10). In some instances the metastasizing thyroid tissue resembled normal gland while in other hyperchromatism, stratification, and frequent mitotic figures were seen.

Physiological and Biochemical Studies. Metabolic rates and protein-bound iodine: (Table 2)

The addition of propylthiouracil to the diet resulted in a fall in the metabolic rate and plasma protein-bound iodine values to hypothyroid levels. In group 2 (propylthiouracil plus iodide) the metabolic rate was even lower, but the protein-bound iodine was above the control value. The values obtained for rats which had received propylthiouracil plus dried thyroid (group 3) were slightly below those of the controls.

TABLE 2. METABOLIC RATE AND PLASMA PROTEIN-BOUND IODINE VALUES

Group	No. of rats	Metabolic rate		No. of rats	Protein-bound iodine	
		Average MR* (15 months)	% decreased from normal		Median, µg.	S.D.
0	16	150	0	13	4.63	±1.17
1	8	99	34	15	1.93	±0.61
2	7	65	57	12	6.93	±1.53
3	8	138	8	10	2.55	±0.62

* Cc. O_2/min./m^2 at N.T.P.

Radio-active iodine uptake:

The uptake of radio-active iodine (24 hours) calculated on a basis of concentration per unit of thyroid tissue was diminished in the rats which had been fed propylthiouracil (group 1). The uptake was less in the rats which had been fed propylthiouracil plus thyroid extract (group 3) and least in those rats which had received propylthiouracil plus iodide (group 2).

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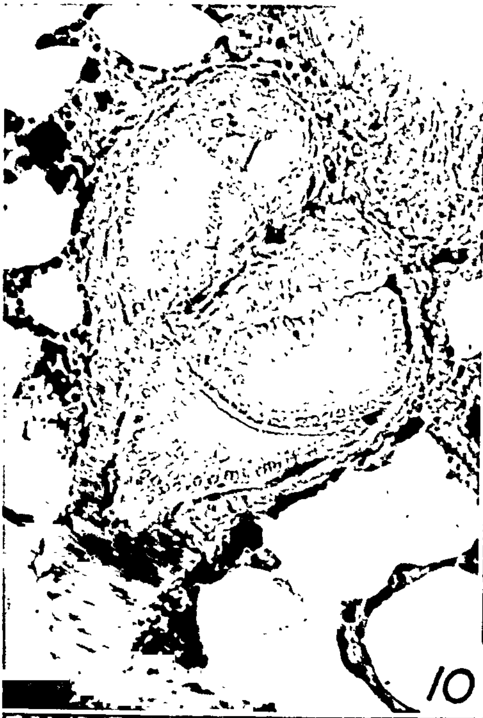
EXPLANATION OF PLATE III

FIG. 10. Lung. Thyroid tissue in a branch of the pulmonary artery. The muscular wall of the vessel is seen in the lower left, and bronchial epithelium adjacent to the artery, in the upper right. (Mallory×150.)

FIG. 11. Pituitary (see Fig. 1). Localized areas of chromophobic cells found in many rats of the propylthiouracil-thyroid group. (Acid fuchsin, methyl blue×40.)

FIG. 12. Pituitary. Edge of a chromophobic area (right). The cytoplasm of the pseudocapsular cells is compressed and stains indefinitely, the nuclei are pyknotic. (Acid fuchsin, methyl blue×500.)

FIG. 13. Pituitary. A higher magnification of chromophobic area shown in Fig. 11. Both cytoplasm and nuclei stain poorly, vary in size and shape. In the photograph red blood cells appear black, one basophil can be seen (upper right). (Acid fuchsin, methyl blue×500.)



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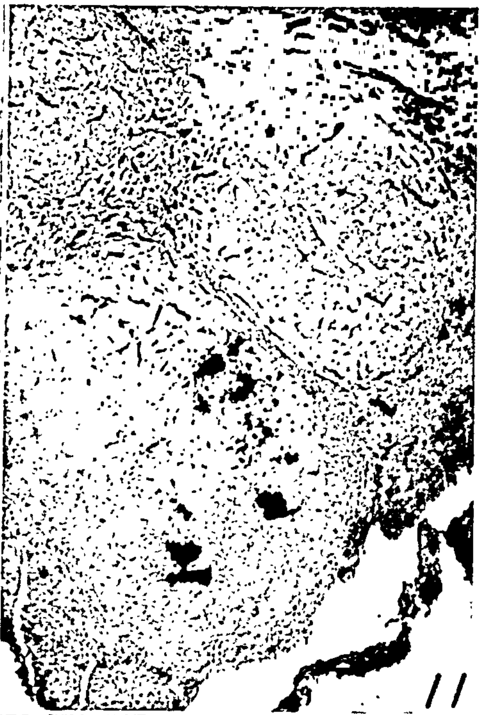

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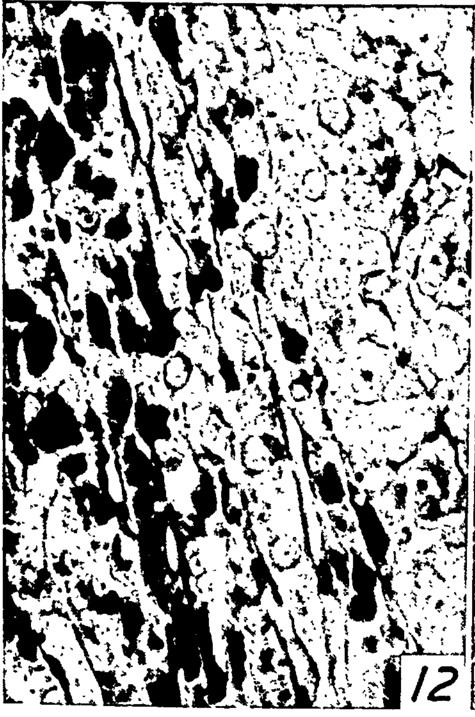

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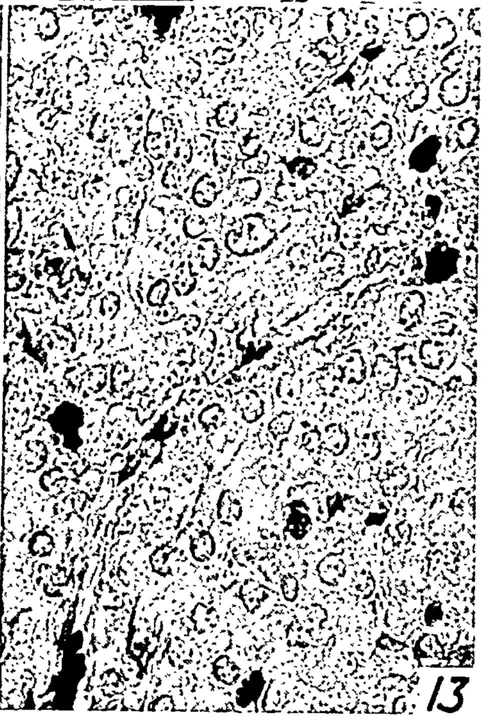

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PLATE III

Although only six animals in each group were studied, the differences were significant. No significant difference existed when the calculation was carried out for total weight of thyroid tissue.

Radioautographs (24 hours) showed little or no concentration in the three groups which received propylthiouracil. The difference between groups, if any, could not be quantitated satisfactorily. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas.

Iodine content of the thyroid gland: (Table 3)

The iodine content of the thyroid gland was very low in the rats which had been fed propylthiouracil (group 1). The level of iodine was higher in those rats that had received propylthiouracil plus iodide (group 2). In the group which had been given propylthiouracil plus thyroid extract the iodine content of the whole thyroid was found to be only one-third less than that of the controls, although the value was very low when calculated per unit weight of thyroid tissue.

TABLE 3. IODINE CONTENT OF THYROID

Group	No. of rats	Iodine content (μg. per 100 mg. wet tissue)		Iodine content (μg. iodine per gland)	
		Median	Range	Median	Range
0	21	130.4	88.4 –196.40	46.6	18.2 –58. 3
1	29	2.41	0.41– 11.40	2.74	1.08– 6.86
2	18	23.68	8.32– 39.89	11.83	7.20–20.40
3	13	5.64	2.60– 10.19	27.68	21.50–43.20

Pituitary

Weights: (Table 1). The pituitaries of the rats which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) were no heavier than those of the control animals. The addition of thyroid extract to the propylthiouracil (group 3) caused a significant increase in the weight of the pituitaries.

Pathology. Two of the pituitary glands from rats which received propylthiouracil plus thyroid extract (and one, the largest of all, from a rat receiving propylthiouracil plus iodide) were large (pea-sized), friable and dark red in color. Most of the glands from this group (Figs. 1 and 2), although somewhat larger than usual, looked normal except that in many instances there were one or more small flattened reddish nodules on the infero-lateral surfaces of the anterior lobe. The larger of these were as big as a normal pituitary (rat).

Microscopic: There was a consistent degranulation of the eosinophil cells in the treated groups which varied in degree from animal to animal. In spite of this it was usually, but not invariably, possible to find some cells which contained eosinophilic granules in part of a section. Basophils were almost always degranulated; and often the cells were enlarged and the cytoplasm was vacuolated. In most sections from animals of treated groups a considerable number of very large cells showing extreme vacuolation of the cytoplasm but no granulation could be seen. The staining characteristics of the cytoplasm varied but usually stained pale blue with basic stains.

There was a relative increase in chromophobic cells.

In a high proportion of the propylthiouracil plus thyroid group, areas which stained very poorly were demarcated from normal tissue by a pseudocapsule of compressed cells (Figs. 11, 12). These areas were made up of masses of "chromophobic" cells, the nuclei of which varied in size and appearance from small, deeply stained, almost pyknotic, to very large hypochromatic structures. In a few sections faint eosinophilic granulation could be made out in the cytoplasm. The cytoplasm was pale, profuse and the cell borders were irregular (Figs. 13, 16, 17). The cytogenesis of the cells is not clear, and in spite of the chromophobic nature of the cells we are inclined to believe that they may have originated from chromophil cells. The evidence for this is not entirely convincing and consists of finding eosinophilic and less often basophilic transitional forms (Figs. 16, 17).

In the large hemorrhagic tumors found grossly, the microscopic appearance showed chromophobic cells interspersed with numerous mast cells (Figs. 14, 15). No "transitional" forms were seen in these instances. Areas of hemorrhage and necrosis were present and these contained deposits of "ceroid" demonstrable by special staining.

Adrenals

Weights: (Table 1). The adrenals of the female rats (but not the male) which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) weighed less than those of the control animals. Those rats which had received propylthiouracil plus thyroid extract had adrenal glands which weighed approximately the same as those of the control animals.

Pathology. The adrenal glands of most of the rats in the groups treated with propylthiouracil, or propylthiouracil plus iodide showed involutionary changes in the zona reticularis (Fig. 18). The addition of dried thyroid to the diet (group 3) prevented this involutionary change.

Exophthalmos

In Table 4 it is seen that a high percentage of animals receiving propylthiouracil became exophthalmic. Dried thyroid reduced the incidence very greatly but iodide was ineffective.

DISCUSSION

Rats which received propylthiouracil plus thyroid extract achieved an almost normal growth curve. This suggests that the depression of growth observed in the rats to which propylthiouracil or propylthiouracil plus iodide was given was due to a lack of thyroid hormone (Hammett, 1926) rather than to a direct inhibitory effect of proplythiouracil on the growth hormone of the anterior pituitary (Williams *et al.*, 1944).

The addition of a relatively large amount of iodide (approximately 1.5 mg. sodium iodide per rat per day) to the diet containing propylthiouracil reduced the goitrogenic effect of propylthiouracil, although the reduction of metabolic rate was even greater than in the group which received propylthiouracil alone.

TABLE 4. EXOPHTHALMOS

Group	No.	Exophthalmos			Incidence (per cent)
		Present	Absent	Doubtful	
0 (control)	25	0	24	1	0
1 (PTU)	35	29	4	2	83
2 (PTU+I)	22	17	4	1	77
3 (PTU+Th)	16	3	12	1	19

Thyroid

An unexpected finding was the tremendous enlargement of the thyroid gland when dried thyroid (approximately 3 mg. per day) was added to the diet containing propylthiouracil. This amount of thyroid had been chosen in an attempt to maintain the growth rate and metabolic rate within nor-

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EXPLANATION OF PLATE IV

FIG. 14. Pituitary, Chromophobic adenoma. Variation in nuclear and cell size is apparent. Nuclear disintegration, extreme vacuolation of the cytoplasm and in other sections hemorrhage and necrosis are seen. The cells which appear black in photograph are mast cells. (Acid fuchsin, methyl blue×500.)

FIG. 15. Pituitary. Chromophobic adenoma. Large purple granules (black in photograph) cover the nuclei of cells having all the staining characteristics and appearance of mast cells. (Basic fuchsin—fast green×1000.)

FIG. 16. Pituitary (gross fig. 2). Chromophobic area with large vacuolated cells, showing no distinctive granulation, stained pale blue with basic stains. (Acid fuchsin, methyl blue×1280.)

FIG. 17. Another field of same pituitary showing transitional chromophil cells. Upper cells (B) are basophils; lower cells (A) are eosinophilic. (Acid fuchsin, methyl blue×1280.)

FIG. 18. Adrenal. At lower right the large pale chromaffin cells are normal medullary tissue. Approximating this area atrophied cells of the reticularis, surrounded by black reticular fibres can be seen. (Mallory×500.)

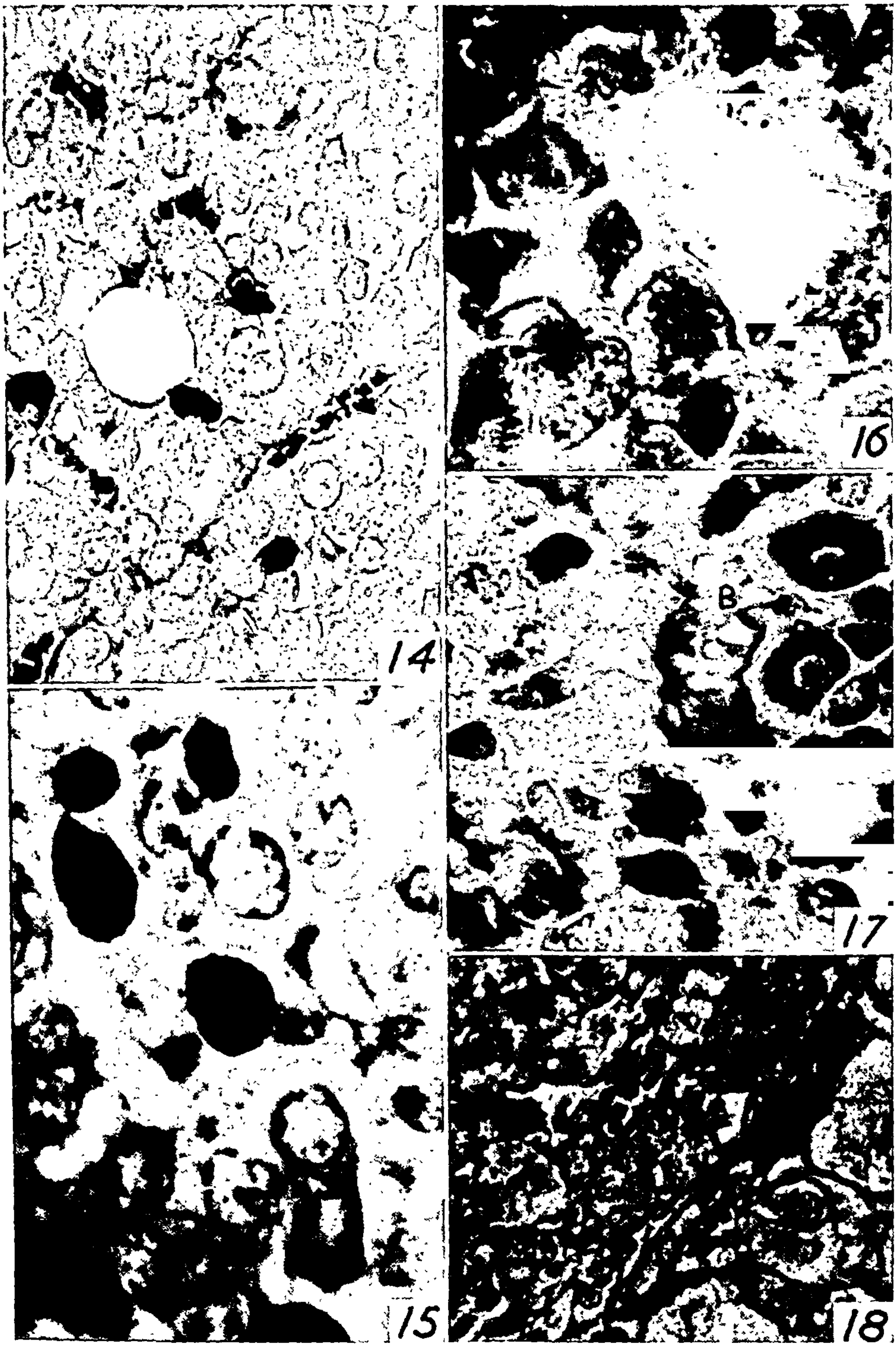

PLATE IV

mal limits in spite of the continued administration of propylthiouracil. Both of these values were slightly lower than those of the control group but fell within the range of "normal." Nevertheless, the size of the thyroid, and of the pituitary was significantly greater than in the other groups. It is necessary to postulate that there was some substance in the thyroid extract which stimulated the pituitary and the thyroid gland, or that the thyroid hormone supplied as dried thyroid in the diet was less than necessary to inhibit the pituitary, and a "hormonal imbalance" existed. Other evidence at hand, showing that in lengthy experiments of this nature, relatively large amounts of crystalline l-thyroxine added to the diet are necessary to prevent enlargement of the thyroid, suggests that the second alternative is more likely. It is possible that the thyroid hormone exerts a stimulatory function (under some circumstances) on the cells of the pituitary and thyroid gland. Unfortunately we have no information on the thyrotropin content of the anterior pituitary under the conditions described here. The great enlargement of the thyroid, and the production of metastasizing adenomas, suggest that continued stimulation of the thyroid gland by the thyrotropic hormone is the important causal factor (Purves and Griesbach, 1949; Money and Rawson, 1950).

The spread of the tumors appears to be through vascular channels. Apart from local invasion of the capsule of the gland, metastases were found exclusively as intact thyroid follicles in blood vessels in the thyroid, in venules adjacent to the thyroid, in branches of the pulmonary artery, and in alveolar vessels. No attempt was made to transplant or culture the tumors, but from other somewhat similar studies (Morris, Dalton and Green, 1951; Hall and Bielschowsky, 1949; Bielschowsky *et al.*, 1949) it would appear that they are at least *potentially* malignant.

Pituitary

The development of chromophobe adenomas of the pituitary in mice following destruction of the thyroid by radio-iodine has been reported by Furth (1952). As far as we know, no investigators have observed such tumors subsequent to the administration of goitrogenic drugs. Beyond compression of the base of the brain, not even the largest showed evidence of infiltration of adjacent tissue, or metastatic spread. The tumors were made up of chromophobic cells of very variable size interspersed with mast cells. Areas of hemorrhage and necrotic tissue (containing ceroid) were present in the larger ones. It has already been observed that in our opinion this staining characteristic does not necessarily mean that they arose from chromophobe cells. Indeed our present view tends to oppose this.

The metabolic rate and protein-bound iodine were lower in the group receiving propylthiouracil than in the controls. The lower metabolic rate obtained when iodide was given in addition to propylthiouracil is difficult to explain but is perhaps related to the relatively high dose level of iodide. The higher value obtained for the protein-bound iodine in this group probably reflects the higher intake of iodide and has been observed by others (Barker, 1948). In the group receiving thyroid the protein-bound iodine value was lower than normal, which supports the hypothesis that the amount of thyroid given was inadequate to replace that normally supplied by the gland.

Iodine content of the thyroid

The low iodine content of the thyroids from animals which received propylthiouracil, and the absence of concentration of iodine shown by radioautography was not unexpected. The differences between groups (in iodine content) are appreciable but cannot be explained satisfactorily from available information. The finding of granular eosinophil cells in the pituitaries of *all* the treated groups would imply (Purves and Griesbach, 1947) that thyroidal or extrathyroidal production of some thyroid hormone was taking place in both the propylthiouracil and propylthiouracil plus iodide groups. If such were the case, differences in iodine take-up and content might be expected with the differing treatments.

Exophthalmos

The production of exopthalmos in rats by feeding propylthiouracil has been reported previously (Sellers and Ferguson, 1949). This finding which occurred in a high proportion of the animals treated with propylthiouracil, or propylthiouracil plus iodide, was much less common in those animals which were given dried thyroid. Without knowing the amounts of thyrotropin produced or released it is not possible to comment on its possible role in the causation of exophthalmos. From the size of the thyroid glands, one would assume that the amount of thyrotropin available was large. Nevertheless, administered thyroid effectively prevented the exophthalmos.

SUMMARY

For a period of 15 months or more Wistar rats were fed the usual laboratory ration supplemented with (1) propylthiouracil, 0.02%, (2) propylthiouracil plus 0.02% sodium iodide, (3) propylthiouracil plus 0.02% dried thyroid.

Growth and metabolic rates were much lower than normal with propylthiouracil or propylthiouracil plus iodide but were nearly normal with propylthiouracil plus thyroid.

The thyroid glands were enlarged greatly in the three treated groups; the differences in gland size between each group (including the control)

were significant. The thyroids of rats which received iodide showed the least enlargement and those of rats which received dried thyroid with propylthiouracil the greatest (up to 80 times normal).

Histologically, in addition to the hyperplasia and disappearance of colloid typical of propylthiouracil administration, circumscribed areas of large follicles containing colloid were present. The cellular detail and apparent "activity" in these adenomas varied greatly. The particular treatment did not appear to control the type of adenoma produced. The radioiodine uptake was low in treated groups. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas (24 hour radioautographs).

In 3 or perhaps 4 cases metastatic thyroid tissue was found in the lungs. Spread was by vascular channels as metastases were found in venules of thyroid, esophagus, muscle, and in a branch of the pulmonary artery. No attempt at transplantation or culture was made.

The pituitaries of rats receiving propylthiouracil plus thyroid were significantly heavier than those of other groups. Single or multiple "chromophobic" adenomas occurred frequently in this group.

The eosinophils of the pituitaries of all treated groups were incompletely degranulated. Generally the basophils were degranulated, large and vacuolated.

It is suggested that the tremendous increase in size of the thyroid gland, the great enlargement and adenomas of the pituitary, in the propylthiouracil plus thyroid group may be ascribed to a hormonal imbalance rather than to some unknown substance in the dried thyroid. The thyroid hormone itself may exert some stimulating function on the cells of the pituitary and thyroid.

Exophthalmos occurred in most (about 80%) of the rats receiving propylthiouracil or propylthiouracil plus iodide. It occurred in about 20% of those receiving propylthiouracil plus thyroid.

The adrenal glands of female rats receiving propylthiouracil, propylthiouracil plus iodide, but not propylthiouracil plus thyroid, were significantly lighter than those of the controls. Histologically the reticularis showed involutionary changes.

Acknowledgment

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BIOASSAY OF RELAXIN

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IN 1926 Hisaw first demonstrated experimentally the relaxation of the pelvic ligaments of guinea pigs during estrus following the injection of blood serum from pregnant rabbits. Later work indicated a specific hormone, designated as relaxin, was responsible for the looseness of the symphysis pubis, since the effect was also observed following injection of extracts of sow corpora lutea into castrated guinea pigs provided the animals were pretreated with estrogen. Since that time, most of the experimental work on relaxin has been based upon bioassays with this all-or-none pelvic looseness endpoint determined by manual palpation. During this assay procedure, the possibility of traumatizing the loosened tissue of the pubic symphysis or exaggerating the hormone-induced separation cannot be excluded. The radiographic measurement of the inter-pubic gap would seem desirable because not only would this provide an objective record but the determination of the actual interpubic distance might provide a more sensitive assay. Hall (1947) and Talmage and Hurst (1950) have employed dental x-ray machines to demonstrate relaxin activity but few details of the actual technics employed have been published. Talmage and Hurst attempted to refine the conventional guinea pig assay by substituting radiographic measurements for manual manipulation. However, the variability of response of individual animals was so great that these investigators concluded the assay would be impractical. The use of x-rays to determine separation of the pubic symphysis seems justified because Hall (1947) has shown that in untreated pregnant mice the time of beginning of pubic separation coincides within a day or two with the first histological evidence of relaxin effects on the pubic tissues.

This paper represents an attempt to develop a more reliable bioassay for relaxin employing mice with radiographic evaluation of the response.

EXPERIMENTAL

Swiss albino virgin mice[1] were employed after ovariectomy, except in later trials when intact immature females were employed. After a five-

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[1] Albino Farms.

day postoperative period, each animal received daily intraperitoneal injections of 1.0 μg. estradiol benzoate in corn oil, usually for a total of eight days. This procedure was varied in some experiments as will be indicated. During the experiments where relaxin was injected over a period of more than one day, the estrogen injections were continued on a daily basis.

In order to standardize the positioning of the animal for radiography as well as to remove the operator from the area of harmful radiation, the holder, illustrated in Figure 1, was constructed.[2] In use, the lower abdomen of the mouse rests directly upon the film. The cephalic edge of the film is tilted upwards into the belly of the animal to approximate a right angle with the x-ray beam. The film left projecting from the rear of the holder conveniently is used for placing the lead numerals to identify the individual negatives. For the accurate radiographing of the inter-pubic gap, the x-ray beam must strike the symphysis sagitally and, in order to avoid the vertebral and tail bones, from some angle below the vertical. After the animal's hindquarters are fixed in the holder with the hinged restrainer bar, the tail is pulled upward and forward and wedged into the forked holder. This motion tilts the entire pelvis and tends to closely approximate the symphysis against the film. The focal spot of the x-ray tube was at least 35 cm. from the animal so that the image on the film could be assumed to represent the actual bony separation. Measurements obtained under these conditions agree closely with figures published by Hall (1948).

It was found that the consistent production of sharp radiographs which permitted accurate measurements was difficult. The x-rays pass through approximately 10–12 cm. of soft tissue and careful exposures were necessary to produce sufficient contrast with the thin bony parts for accurate visualization of the pubic gap. Since the sole aim of the x-ray technic was the production of an objective record of the separation of the pubic bones, the use of high kilovoltages, customarily employed in routine medical radiographic work to bring out detail, resulted in films with such gradual density transitions across the pubic symphysis that the precise placing of the caliper points was impossible. The quality of radiographic image required was the short-scale, high contrast type. The x-ray machine available for this work was a General Electric Portable, Model F, with a 10 milliampere capacity and a constant output of 63 kilovolts. By interposing a injecting the animals twice per day it was found that an 8–16 hour schedule was equivalent to a 12-12 hour schedule, and when injections were continued for periods up to nine days the maximum separation occurred at 2–5 days with increasing variable receptivity to relaxin. Therefore an assay period of 48 hours was selected as representing the time of optimal response. In an additional experiment the pubic response to four doses of relaxin administered at 12-hour intervals was compared to that of six doses given at 8-hour intervals. The data are presented in Figure 2. The total amount injected in each group was 1.0 G.P.U. per day and no significant difference was apparent. Therefore an 8-16-8-16 hour, twice per day for 48 hours, injection schedule for all assays was adopted.

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[2] The holder was made from 0.250″ sheet of Plexiglass with the hinged restrainer bar bent to shape by the cautious application of heat. An ordinary snap fastener of the type usually employed on canvas awnings was used to keep this bar fastened down when in use. The film slot was built up of wedge-shaped pieces to position the dental films approximately 20° from the horizontal. Two thin rabbeted pieces were cemented to the edges of the inclined wedge to accommodate dental films inserted with the long diameter parallel wth the holder.

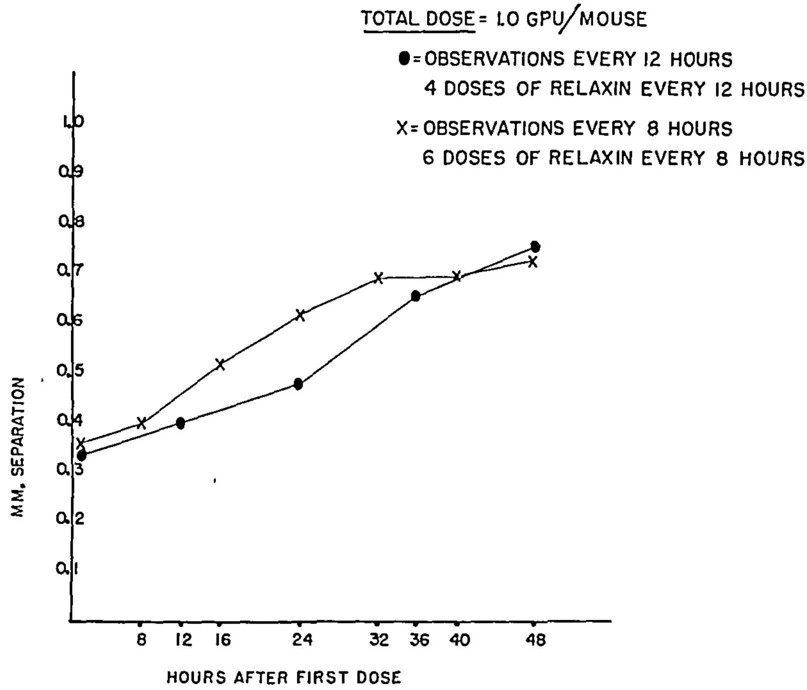

FIG. 2.

In a few experiments, treating the mice with ACTH, cortisone, progesterone, or desoxycorticosterone in addition to estrogen and relaxin resulted in no increment in response. Control groups pre-treated with estrogen and injected with saline showed no response.

Before each assay, a control radiograph was taken immediately prior to the relaxin injection. The average pubic separation of both ovariectomized and intact, immature female mice, as determined after estrogen pretreatment but prior to relaxin administration, ranged from 0.32 to 0.35 mm. About 15% of the ovariectomized animals had an initial pubic separation of over 0.5 mm., and although experience showed that these particular animals responded with an equal or greater relative separation than the remaining animals, they were arbitrarily discarded in order to reduce assay variation.

The pubic responsiveness to relaxin was not influenced by the age at which ovariectomy was performed between 30 and 73 days of age. No significant difference in response could be detected between the following varying conditions: Administration of estrogens subcutaneously and of relaxin intraperitoneally, both hormones administered subcutaneously, and finally the estrogen administered intraperitoneally and the relaxin subcutaneously.

TABLE 1. INFLUENCE OF NUMBER OF DAYS OF ESTROGEN PRETREATMENT ON PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:
Relaxin injections—2 per day for 2 days.
Observations—Pre-relaxin, 24 hours and 48 hours post-relaxin.
Estrogen—1 μg./day estradiol benzoate in corn oil.
Relaxin—0.5 G.P.U./day.

No. of estrogen inj. before relaxin treatment	No. of mice	Mean weight gm. ±S.E.	Pelvic separation		
			Pre-relaxin mm.	Post-relaxin 24-hrs. mm.	Post-relaxin 48-hrs. mm.
4	16	28 ±0.5	0.33 ±0.01	0.50 ±0.66	0.74 ±0.07
5	30	28 ±0.5	0.32 ±0.01	0.53 ±0.05	0.74 ±0.06
6	7	30 ±0.9	0.34 ±0.02	0.56 ±0.11	0.76 ±0.21
6	16	29 ±0.7	0.32 ±0.01	0.48 ±0.07	0.65 ±0.10
6	15	26 ±0.4	0.36 ±0.02	0.49 ±0.04	0.65 ±0.08
6	16	28 ±0.7	0.35 ±0.01	0.50 ±0.04	0.68 ±0.08
7	9	28 ±1.0	0.32 ±0.01	0.45 ±0.03	0.59 ±0.09
8	15	27 ±0.8	0.33 ±0.02	0.46 ±0.05	0.67 ±0.10

Estrogen pretreatment was varied from four to eight days as illustrated in Table 1. No significant difference in response could be observed.

An attempt was made to use the male mouse for the assay. As Table 2 illustrates, no significant pelvic response could be elicited even when doses as high as 1.2 G.P.U. per day were administered.

Attempts to establish dose-response curves are recorded in Tables 3 to 6. In Table 3 the response of increasing doses of relaxin from 0.15 G.P.U. to 0.60 G.P.U. per day was studied with an estrogen pretreatment period of six days. As little as 0.15 G.P.U. total dose produced a significant response in 24 hours, but when the dose within the day period, was increased to 0.30 or 0.60 G.P.U. only small increments in response were apparent. When the assay was run for two days, a relatively large difference between 0.15 G.P.U. per day and 0.30 G.P.U. per day was found. Increasing the dose to 0.60 G.P.U. per day in the two-day assay did not result in a significant increase in response. The data were also used in a

2×2 design according to Bliss (1944). Nine animals were used at each concentration of 0.15 and 0.30 G.P.U. per day. A potency ratio of 126.6% was found. The error at P = 0.95 was +252% and −63%. The t value was 0.640 and the index of λ was equal to 0.426.

TABLE 2. MALE MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:

Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R**	E R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 48 hrs. mm.
0.15	13	28 ±0.7	0.32 ±0.01	0.32 ±0.01	0.32 ±0.01
0.30	15	26 ±0.6	0.32 ±0.01	0.33 ±0.01	0.34 ±0.01
0.60	16	28 ±0.6	0.32 ±0.01	0.36 ±0.02	0.46 ±0.05
1.20	20	29 ±0.7	0.30 ±0.01	0.34 ±0.01	0.34 ±0.01

*E = Estradiol benzoate in corn oil 1 μg./day.
**R = Chilcott Releasin 31-81D SF.

TABLE 3. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:

Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R** R	E R R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 4-hrs. mm.
0.15	19	28 ±0.5	0.33 ±0.01	0.41 ±0.03	0.57 ±0.04
0.30	30	28 ±0.6	0.33 ±0.01	0.53 ±0.05	0.91 ±0.10
0.60	13	31 ±2.4	0.33 ±0.02	0.55 ±0.10	0.93 ±0.12

* E = Estradiol benzoate in corn oil 1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Nine.
$X = 100$ dose per day.
$Y = 1.16\ (X - 1.36) - 0.78$.
Doses from 0.15 to 0.30 G.P.U./day.

Additional dose-response data are presented in Tables 4, 5, and 6. In Tables 4 and 5, immature intact female mice were used in place of ovariectomized animals. When two doses of estrogen were employed daily (Table 5) a reasonably linear response was observed over the range 0.05 to 0.50 G.P.U. per day. With one dose of estrogen per day, the useful range was 0.05 to 0.25 G.P.U. per day.

Table 6 summarizes the dose-response data using ovariectomized females with an estrogen pretreatment period of five days. The slope of 0.60

is favorable and the useful range appears to be between 0.10 to 0.80 G.P.U. per day.

The utility of the method (see Table 6) was further tested by the simplified 2×2 design of Bliss. Two concentrations of each of the unknown and standard were 0.25 and 0.50 G.P.U. per day. Since the same material was used for both the unknown and the standard, the theoretical potency ratio was 100. When ten animals were used at each concentration for both unknown and standard a potency ratio of 77% was found. The t value for

TABLE 4. RELAXIN IN IMMATURE, INTACT FEMALES (28 DAYS AT START)

Injection—observation schedule:

Days	1	2	3	4	5	6
Treatment	E*	E	E	E R** R	E R R	E
Observation				O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Pelvic separation Day 4 pre-relaxin mm.	Pelvic separation Day 5 post-relaxin mm.	Day 6 post-relaxin mm.
0	8	16±0.9	0.34±0.01	0.34±0.01	0.34±0.02
0.05	13	18±0.5	0.35±0.01	0.40±0.02	0.38±0.03
0.10	14	17±0.4	0.32±0.01	0.48±0.04	0.51±0.06
0.25	17	16±0.7	0.36±0.01	0.53±0.05	0.61±0.05
0.50	18	16±0.6	0.33±0.01	0.47±0.03	0.56±0.05

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D SF.
Data of Day Six
$X=100$ (dose per day).
$Y=0.57\ (X=1.07)+0.51$.
Doses from 0.05 to 0.25 G.P.U./day.

TABLE 5. MOUSE PELVIC RESPONSE TO RELAXIN IN IMMATURE, INTACT, FEMALES (28 DAYS AT START)

Injection—observation schedule:

Day	1	2	3	4	5	6
Treatment	E* E	E E	E E	E+R** E+R	E+R E+R	
Observation				O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ±S.E.	Day 4 pre-relaxin mm.	Day 5 post-relaxin mm.	Day 6 post-relaxin mm.
0.05	21	17±0.4	0.32±0.01	0.34±0.01	0.37±0.01
0.10	18	17±0.8	0.32±0.01	0.40±0.03	0.50±0.06
0.25	20	17±0.04	0.33±0.01	0.53±0.05	0.71±0.07
0.50	40	17±0.04	0.35±0.01	0.69±0.06	0.84±0.08

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D AF.
Data 6
$X=100$ (dose per day).
$Y=0.412\ (X-1.30)+0.65$.
Doses from 0.05 to 0.50 G.P.U./day.

significance of difference of slopes was 0.915 showing that the slopes were not significantly different, and at P = 0.95, the expected error determining the potency ratio was −49% and +96%. When the number of animals was increased to 16 in each group, the potency ratio was found to be 91.5% and the error at P = 0.95 was −35 to +55%. The index of precision λ was equal to 0.375. The t test for differences between slopes was 0.673 which means that the slopes of unknown and standard were not significantly different.

TABLE 6. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule								
Days	1	2	3	4	5	6	7	8
Treatment	E*	E	E	E	E	E R** R	E R R	
Observation						O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ±S.E.	Pelvic separation Day 6 prerelaxin mm.	Pelvic separation Day 7 post-relaxin 24 hr. mm.	Pelvic separation Day 8 post-relaxin 48 hr. mm.
0	21	29 ±0.6	0.30 ±0.01	0.33 ±0.03	0.29 ±0.01
0.10	17	27 ±0.5	0.30 ±0.01	0.33 ±0.03	0.37 ±0.05
0.25	19	26 ±0.6	0.31 ±0.01	0.42 ±0.04	0.50 ±0.05
0.50	30	28 ±0.5	0.32 ±0.01	0.53 ±0.05	0.74 ±0.06
0.60	21	30 ±0.6	0.33 ±0.01	0.49 ±0.06	0.77 ±0.09
0.80	12	31 ±0.5	0.30 ±0.01	0.76 ±0.14	1.03 ±0.13
1.00	7	28 ±0.9	0.37 ±0.02	0.66 ±0.10	1.10 ±0.20
4.00	9	28 ±0.6	0.33 ±0.02	0.87 ±0.18	1.20 ±0.24

* E = Estradiol benzoate in corn oil—1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Eight
$X = 100$ (dose per day).
$Y = 0.66\ (X - 1.57) + 0.68$.
Doses from 0.10 to 0.80 G.P.U./day.

DISCUSSION

One of the major advantages of employing mice for the bioassay of relaxin, aside from the obvious advantage of housing small animals, is that the animals are used only once and then discarded. In this way the sensitivity of the assay animal tends to be constant and the sensitizing effects of previous courses of estrogen and relaxin are avoided. The effect of employing the same animals for more than a single assay was effectively demonstrated when a group of nine animals were given the usual assay treatment of six days pre-treatment with estrogen plus two days estrogen and relaxin at 0.5 G.P.U. per day. The mean pubic separation increased from 0.33 mm. ±0.02 to 0.86 mm. ±0.15. After a five day rest period these animals were again used. This time the mean pubic separation was

0.84±0.17 at zero hours and increased to 1.44±0.24 at the end of 48 hours. This tendency to increased sensitivity to estrogen and relaxin following a previous pregnancy or assay use has been observed by various workers and represents a serious drawback in the conventional guinea pig assay where the animals are used repeatedly without a rest period of at least 7 to 10 days.

Talmage and Hurst (1950) attempted to refine the traditional guinea pig palpation assay by the radiographic measurement of the interpubic distance, but apparently the guinea pig pelvis separates less than 50 per cent over the resting gap as determined by these workers radiographically. According to their published figures the average interpubic distance determined before relaxin administration among their assay animals was between 0.75 and 1.0 mm. Following the injection of relaxin, there was an average increase of approximately 0.5 mm., but the great variability of response not only between individual animals, but between similar doses in the same animal, was so large that these observers concluded at least 50 animals at each of three dose levels would be necessary to provide a valid assay. They also observed a failure to return to the pre-relaxin measurement in about 10 per cent of pigs used for repeated assays, and a significant number of pigs repeatedly employed for relaxin assay sooner or later became permanently relaxed and had to be discarded.

The superiority of mice as compared to guinea pigs for relaxin assay is undoubtedly related to the dramatic separations which can be produced in the mouse by artificial means. Although we were unable to completely reproduce the separations observed in normal pregnant mice, this objective was more nearly attainable in mice than in guinea pigs. When 10 G.P.U. of relaxin were administered daily in two divided doses for eight days, the pubic gap increased from 0.29 mm. ±0.02 to 2.49±0.30. This was the maximum increment in pelvic gap produced by relaxin, and represented more than 800 per cent of the resting gap. It is probably true that the pubic separation occurring in pregnant mice undoubtedly represents an optimal hormonal environment culminating in a peak separation at term. Hall (1946) has shown that injections of estrone and/or progesterone into intact pregnant mice do not increase the observed separation.

In most of the experiments presented in this paper the mice were selected for assay on the basis of body weight. These animals were therefore of different ages and in order to determine the effect of age upon the individual sensitivity to relaxin, several experiments were performed with groups of animals of various known ages (30 to 73 days). No consistent effect attributable to age could be ascribed and at this time the effect of age prior to ovariectomy cannot be evaluated.

Recent work has been with unoperated intact immature female animals.

The pre-relaxin baseline separation in these animals, as compared with ovariectomized mice, was relatively more constant and only an occasional animal was discarded because the gap measured over 0.5 mm. This assay technique is apparently practical and although the maximum response seems less than with ovariectomized animals, an attempt is being made to determine its possible value.

SUMMARY

A sensitive, convenient and practical bioassay for relaxin is presented employing mice, with radiographic measurement of the pubic separation.

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BIOCHEMICAL PROPERTIES OF FRACTIONS OBTAINED FROM RAT ANTERIOR PITUITARY GLANDS BY DIFFERENTIAL CENTRIFUGATION[1]

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THE biochemistry of the nuclear, mitochondrial, microsomal and supernatant fractions isolated by differential centrifugation from rat and mouse liver has been studied extensively during the past few years. Particular attention has been given to the distribution of the oxidative enzymes and nucleic acids among these fractions. Respiratory enzymes such as cytochrome oxidase and the succinoxidase system have been found to be associated with the mitochondrial fraction (Schneider, 1946; Hogeboom *et al.*, 1946 and 1948). The microsomal fraction contains few enzymes but as much as 50% of the pentosenucleic acid of liver tissue (Hogeboom *et al.*, 1948; Schneider *et al.*, 1950.) The results of studies of this nature have been summarized by Schneider and Hogeboom (1951).

Little attention has been given to the biochemical and hormonal properties of fractions of endocrine tissues isolated by differential centrifugation. Approximately 60% of the succinoxidase activity of rat anterior pituitary glands, however, has been reported to be associated with a large granule fraction (McShan and Meyer, 1949), and 75% of the gonadotrophic hormone activity with a small granule fraction (McShan and Meyer, 1952). The purpose of this paper is to describe a study of the gonadotrophic hormone activity of fractions obtained from anterior pituitary glands of adult normal and castrate rats as related to the succinoxidase, glycolytic and transaminase activities; the desoxypentose-nucleic acid (DNA) and pentosenucleic acid (PNA); and the nitrogen or protein contents of these fractions.

MATERIALS AND METHODS

The anterior pituitary glands of normal and castrate adult female rats of the Holtzman-Rolfsmeyer strain were used for fractionation. The minimum time of castration before using the animals as donors was three weeks and the maximum time was six months.

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The use of rats for experiments of this kind is advantageous as the glands can be removed and homogenized within a few minutes after the animals are killed. This allows little time for tissue changes to occur.

Groups of 100 to 150 rats were used for each run. The pituitary glands were removed in the cold room immediately after killing the animals, and kept in a humidor during the short time required to complete removal of all the glands. The anterior lobes were separated, weighed and macerated in a sharp pointed homogenizing tube with 0.88 M sucrose. The volume of the sucrose in milliliters was 19 times the weight of the glands in grams. The homogenates were prepared by mashing the glands instead of grinding since less gonadotrophin is found in the supernatant fraction when the former procedure is followed (McShan and Meyer, 1952).

The homogenates (WH) were differentially fractionated in the multispeed head of an International refrigerated centrifuge at 2° C. by centrifuging at 275, 4,400 and 20,400 g. to give, respectively, the nuclear (NF), large granule (LG), small granule (SG) and supernatant (S3) fractions according to the procedure of McShan and Meyer (1952). The saline extract of the SG fraction was indicated by SGE and the extracted granules by SGR.

The homogenates and fractions were assayed for gonadotrophic hormone activity by use of 21 day old female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with normal saline so that each rat received a total of 20 mg. equivalent of fresh tissue in nine injections of 0.5 ml. each. The first injections were made on the afternoon of the first day followed by injections on the morning and afternoon of the next four days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded.

The homogenates and the fractions were stained for mitochondria by use of fast green FCF as reported by Harman (1950) and the nitrogen determinations were done by a modification of the micromethod reported by Johnson (1941).

The procedure for the preparation of filtrates for the nucleic acid determinations was based on the method reported by Schneider (1945a). The exact procedure employed was as follows: The sucrose homogenates and fractions were mixed with one-half volume of 20% trichloracetic acid (TCA), centrifuged at 2,500 RPM in the cold (2° C.) and the supernatant liquid decanted. The precipitates were suspended in 1 to 2 ml. of water to which was added an equal volume of 10% TCA followed by centrifugation. The supernatant liquid was decanted and the precipitates were washed once more with 5% TCA.

The above precipitates were suspended in 1 ml. of water to which was added 3 ml. of 95% ethyl alcohol. This mixture was centrifuged at room temperature followed by decantation of the supernatant liquid, and a second washing with alcohol.

The alcohol precipitates were mixed with 3 ml. of 3:1 ethyl alcohol (95%)—ether mixture, centrifuged and the supernatant liquids decanted. The precipitates were mixed with 2 to 5 ml. of 5 per cent TCA depending on the amount of tissue and heated at 90° C. for 15 minutes, centrifuged and the clear TCA extracts decanted into graduated 15 ml. centrifuge tubes. The precipitates were washed once with 1.5 ml. of 5% TCA, and centrifuged. The washings were mixed with the above extracts and the total volumes were recorded.

The PNA content of the above TCA extracts was determined by the orcinol method of Mejbaum (1939) as recommended by LePage and Umbreit (1945). DNA was determined by the diphenylamine reaction of Dische (1930) as modified by Schneider (1945).

As a basis for calculating the amounts of DNA and PNA present in the homogenized tissues and fractions certain analytical constants for purified samples of these acids were determined according to Schneider (1945a, 1945b). Constants found for the Beckman Model DU spectrophotometer were Fa = 0.01878 (diphenylamine), Fo = 0.1344 (orcinol), and C = 0.01216. These constants were used for calculating the results since the determinations were made with the Beckman instrument. The percentage values used for phosphorus were 0.0954 for PNA and 0.0992 for DNA. A sample calculation using constants for the Evelyn colorimeter is given in the report by McShan, *et al.* (1950).

The succinoxidase was determined by the method of Schneider and Potter (1943), anaerobic glycolytic activity by the method of Novikoff, Potter and LePage (1948), and transaminase activity by the method of Ames and Elvehjem (1946) as modified by Copenhaver, Shipley and Meyer (1951).

RESULTS AND DISCUSSION

The results of the biochemical studies of anterior pituitary tissue from normal and castrate female rats are summarized in Table 1. The gonadotrophic hormone activity of glands from castrate rats was much greater than that of those from normal animals, and the major part of the activity of both kinds of glands was recovered in the small granule (SG) fraction. Essentially all the gonadotrophin in the SG fraction of castrate females was removed by extracting eight times with 0.9% NaCl solution. This extract was designated as SGE. The extracted granules (SGR), and the NF and LG fractions contained little activity whereas the final supernatant fraction S3 contained gonadotrophic activity sufficient to produce ovaries that had an average weight of 55 mg.

Essentially all the DNA of the glands from normal and castrate rats was recovered in the nuclear fraction (NF). The DNA per whole gland was 71.2 μg. for normal and 70.0 μg. for castrate glands (Table 2). On the basis of the assumption that the DNA content of the pituitary cells is constant (Boivin *et al.*, 1948; Swift, 1950), the castrate glands contained about the same number of cells per unit weight as the normal glands. The average weight of the anterior pituitary glands was 10.4 mg. for normal and 11.1 mg. for castrate rats. The ratio of gland weight to weight of DNA per gland was 146 for normal and 159 for castrate animals. This ratio is an expression of relative cell weight and, on the assumption that the specific gravities are the same, it indicates that at least some cells of the castrate glands were larger than those of normal glands. This is supported by the well known fact that the basophils undergo hypertrophy after castration.

The protein values are given in terms of mg. of protein per 20 mg. of fresh tissue (Table 1). These values for protein (N × 6.25) are equivalent to the total nitrogen of the various fractions and are, therefore, maximum values since a small amount of nonprotein nitrogen was presumably present. The protein value for WH of glands from normal rats was 15% greater than was that for castrate animals. The values for the LG, SG and

Table 1. Biochemical properties of fractions obtained from homogenates of normal and castrate adult female rats

Activity or compound	Units (mg. FT)[1]	Kind of rats[2]	Pituitary fractions								
			WH	NF	LG	SG	SGR	SGE	S3	RCF[3]	Recovery %
Assay—av. wt. ovaries (mg.)[4]	20	N	32 (9)[5]			25 (9)					
	20	C	201(18)	35(6)	36(15)	159(15)	25(17)	145(21)	55(15)		
Protein (N×6.25)	mg./20 mg.	N	3.56(4)[6]	1.03(4)	0.38(4)	0.54(4)	0.23(4)	0.27(2)	1.62(4)		100
		C	3.05(6)	1.01(6)	0.30(6)	0.45(4)	0.20(6)	0.21(6)	1.56(6)		109
Nucleic acid PNA	mg./100 gm.	N	343.6(4)						155.8(5)		
		C	369.4(6)						150.0(6)		
DNA	mg./100 gm.	N	685.2(5)	691.0(6)							101
		C	630.4(5)	602.2(6)							96
Glycolysis	cmm. CO_2/ 20 mg./10 min.	N	25.0(3)	7.0(3)	2.6(3)	1.4(3)			9.5(3)	17.2(3)	82
		C	21.3(3)	5.9(3)	3.1(3)	2.1(3)			(9.8(3)	21.9(3)	103
Transaminase	cmm. CO_2/ 20 mg./10 min.	N	162.0(1)						116.0(1)		
		C	149.4(4)						125.4(4)		
Succinoxidase	cmm. O_2/ 20 mg./10 min.	N	16.3(3)	3.9(3)	4.2(3)	5.4(3)			0.23(3)	16.6(3)	102
		C	14.3(5)	2.7(5)	6.5(5)	1.8(4)			0.50(4)	15.3(4)	107

[1] Milligrams of fresh tissue unless indicated otherwise.
[2] N, normal; C, castrate rats.
[3] Recombined fractions.
[4] Average weight of ovaries from untreated animals of the same age is 13 mg.
[5] Number of animals used for assays.
[6] Number of determinations on which the average value is based.

TABLE 2. DESOXYPENTOSE NUCLEIC ACID CONTENT OF NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Kind of rats	Normal	Castrate
DNA per 100 gm. tissue (mg.)	685.2(5)[1]	630.4(5)
Weight of gland (mg.)	10.4(768)[2]	11.1(958)
DNA per gland (µg.)	71.2	70.0
Gland weight (µg.)/DNA per gland (µg.)	146.0	159.0

[1] Number of determinations on which average value is based.
[2] Number of rats on which average is based.

SGE fractions of glands from normal animals were slightly greater than the values for these fractions of glands from castrate rats. More extractable protein (SGE) was associated with the SG of normal than with those of castrate glands, but the SGR fractions of both normal and castrate glands contained essentially the same amount of protein, namely, 0.23 mg. and 0.20 mg., respectively. Results given in Table 3 show that the protein per gland and the relative amount per cell recovered in the LG, SG and SGE fractions were slightly greater for normal than for castrate glands even though the SG and SGE fractions from the castrate glands contained much more gonadotrophic activity. This means that per unit weight of protein the small granule fraction of glands from castrate rats was much richer in gonadotrophin than the same fraction from glands of normal animals.

The fact that only a slight increase occurred in the total PNA on castration (Table 1) indicates, according to Caspersson (1947), little increase in pituitary protein during castration. Synthesis of small amounts of protein for purposes such as the production of gonadotrophin could have conceivably occurred. However, the possibility should not be overlooked that on castration gonadotrophin was formed from protein already present. It is not clear, of course, as to whether the gonadotrophin was produced by the particules with which it was associated or was concentrated in these particles after production at some other point in the cell.

Essentially 50% of the PNA of the homogenates was recovered in

TABLE 3. CONCENTRATIONS OF PROTEIN IN FRACTIONS FROM NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Compound	Kind of rats	Fraction						
		WH	NF	LG	SG	SGR	SGE	S3
Protein per gland (µg.)	N	1850	540	198	280	120	140	840
(N×6.25)	C	1690	560	166	250	116	115	866
Protein per gland (µg.)[1]	N	26.0	7.58	2.78	3.93	1.72	1.97	11.80
DNA per gland (µg.)	C	24.7	8.00	2.40	3.60	1.66	1.64	12.40

[1] Relative measure of protein per cell.

the S3 fraction. PNA was also found in each of the other fractions but these data are not considered to be reliable from the quantitative standpoint because small amounts of tissue were used for the determinations and the recoveries were not satisfactory. These data are not included. The PNA values for the homogenates were lower than found previously for aqueous homogenates of pituitary glands from young adult rats.

The glycolytic activity of the homogenates of glands from normal rats was slightly greater than the activity of those of castrate rats (Table 1). The NF of both kinds of glands contained more activity than the LG and SG fractions. Almost 50% of the glycolytic activity was found in the supernatant fraction S3 of both the normal and castrate glands which is similar to the results reported by LePage and Schneider (1948) for the soluble fraction of rabbit liver. The SG fractions from both normal and castrate glands were low and not significantly different in glycolytic activity whereas these fractions from normal glands were low and those from castrate glands were high in gonadotrophic activity.

A high percentage of the transaminase activity from both kinds of glands was recovered in the S3 fraction.

The succinoxidase activity of the homogenates of normal glands was 16.3[2] as compared with 14.3 for castrate glands. Satisfactory recovery of activity was obtained when the various fractions were recombined as shown by values of 16.6 and 15.3, respectively, for normal and castrate glands. The NF, LG and SG fractions obtained from both kinds of glands by the procedure referred to above (1952) contained succinoxidase activity. This suggested that these fractions also contained mitochondria and this was shown to be the case by staining. The number present in each of these fractions was not estimated quantitatively. The impression was gained from the staining that mitochondria of varying sizes were present. The fact that they were found in the fractions recovered at different speeds of centrifugation is also indicative of this.

The succinoxidase value for the SG fraction of normal glands was three times the value for castrate glands, namely, 5.4 to 1.8 (Table 1). The latter value for glands of castrate rats is an average of three very low and one high value. The difference between these two values for succinoxidase activity is probably not of any particular significance.

The fact that the LG fraction contains little gonadotrophic activity indicates that gonadotrophin is not associated to a significant degree with the mitochondria of this fraction. On the basis of this it was suggested (McShan and Meyer, 1952) that the gonadotrophin of the SG fraction was not associated with the mitochondria of this fraction, but this explanation does not seem valid since it is conceivable that the gonadotrophin could be associated with the small mitochondria or with other granules contained in the SG fraction. It was reported previously (McShan and Meyer, 1952) that little gonadotrophin is associated with the material recovered from the S3 fraction of an 0.88 M sucrose homogenate by centrifuging at 150,000 g. which, according to the work of Hogeboom, Schneider and Pallade (1948), would be expected to contain submicroscopic particles.

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[2] Cu. mm. of oxygen uptake per 20 mg. of fresh tissue per 10 minutes.

SUMMARY AND CONCLUSIONS

Anterior pituitary glands from normal and castrate adult female rats were homogenized in 0.88 M sucrose and separated by differential centrifugation into nuclear, large granule, small granule and supernatant fractions. The gonadotrophic activity, the protein, PNA and DNA contents, and the glycolytic, transaminase and succinoxidase activities of the homogenates and fractions of both kinds of glands were studied.

The high level of gonadotrophin found in the castrate as compared with the normal glands was concentrated in the small granule fraction, and it was removed from this fraction by extraction with isotonic saline. The distribution of protein in the two kinds of glands was studied. The granule fractions and the extract of the small granules of the castrate glands contained slightly less protein than the same fractions of normal glands.

The DNA was recovered in the nuclear fraction and approximately 50 per cent of the PNA in the supernatant fraction. Castration caused little change in the DNA and PNA.

The glycolytic activity was essentially the same for the homogenates and fractions obtained from glands of normal and castrate rats, but the gonadotrophic hormone activity was much greater in the small granule fraction of glands from castrate as compared with normal animals. The major part of the transaminase was found in the supernatant fraction. The succinoxidase was distributed in the nuclear and granule fractions of both kinds of glands. These fractions as indicated by staining were found to contain mitochondria.

On the basis of these results and those reported previously it appears that though gonadotrophin is not associated to a significant degree with the mitochondria of the large granules nor with the submicroscopic particles recovered by high speed centrifugation of the supernatant fraction, it may be associated with those of the small granules.

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RESPONSE OF PREPUTIAL AND ADRENAL GLANDS OF HYPOPHYSECTOMIZED RATS TO ACTH[1]

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THE growth response of the preputial glands of the female rat to ACTH in adrenalectomized-ovariectomized animals indicates that these glands are subject to the direct action of this trophic hormone (Jacot and Selye, 1952). Furthermore, it has been shown that the ascorbic acid of male rat preputial glands can be depleted by stress or ACTH in acute experiments, and that these alterations in the vitamin content resemble the changes which occur in the adrenal gland following these same stimuli (Hess *et al.*, 1952).

It has been known for some time that the level of adrenal ascorbic acid is greatly reduced by hypophysectomy (Leblond and Chamorro, 1940; Bowman *et al.*, 1940). Tyslowitz (1943) reported that the concentration of ascorbic acid in the adrenal glands gradually declines for about 18 days following the operation. Thereafter, he found that the concentration remained stabilized at about 60% of the value for intact animals. To date, there is no information concerning the concentration of ascorbic acid in the preputial glands of the hypophysectomized rat. The present studies were undertaken to determine the effect of hypophysectomy, together with acute and chronic administration of ACTH, on the weight and ascorbic acid content of the preputial glands, as compared to the adrenal glands of the rat.

METHODS

Twenty-nine male rats of the Holtzman strain were hypophysectomized at the age of 33 days. During the postoperative period they were maintained on laboratory chow and 5% sucrose solution. At the time of autopsy the sella turcica was examined for hypophyseal fragments with the aid of a dissecting microscope. The absence of any such fragments, together with a sustained loss of body weight and a marked loss of testis and ventral prostate weight was considered satisfactory evidence of the completeness of the operation.

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Experiment I. At the end of 3 weeks following hypophysectomy the left preputial and left adrenal glands were removed from 14 animals under nembutal anesthesia. Ten of the animals were then injected with 10 μg. ACTH,[3] via the tail vein, and after one hour the right preputial and right adrenal glands were removed. The glands were freed of excess adipose and connective tissue, weighed on a torsion balance, and subjected to ascorbic acid analysis in accordance with the method of Roe and Kuether (1943).

Experiment II. Two weeks after hypophysectomy, the left preputial and left adrenal glands were surgically removed from 15 animals. Following this operation, the animals were given subcutaneous injections of 25 μg. ACTH twice daily for 7 days. At autopsy the right preputial and right adrenal glands were removed, weighed and analyzed for ascorbic acid content.

Twenty-three intact animals of the same age and sex were used as controls for these experiments. In addition to weights and ascorbic acid values for preputial and adrenal glands, weights for the left testis and ventral prostate were obtained from these animals.

RESULTS

Experiment I. Three weeks after hypophysectomy there is an absolute as well as a relative decrease in the weight and ascorbic acid content of the preputial and adrenal glands (Table 1). On a relative weight basis the

TABLE 1. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE ADRENAL AND PREPUTIAL GLANDS OF MALE RATS

	No. of Rats		Adrenal Glands				Preputial Glands			
			Weight		Ascorbic Acid Content		Weight		Ascorbic Acid Content	
			Abs	Rel.[2]	Abs.[3]	Rel.[4]	Abs.	Rel.[2]	Abs.[3]	Rel.[4]
			mg.	mg.			mg.	mg.		
Control	10	L	13.9 ±0.30*	8.1 ±0.26	60 ±2.6	439 ±18	39.3 ±5.0	22.7 ±2.8	22 ±3.3	57 ±6.3
	10	R	13.1 ±0.22	7.6 ±0.20	60 ±2.2	456 ±16	32.4 ±4.4	18.6 ±2.4	20 ±3.1	59 ±6.8
Hypophysect. (3 weeks)	14	L	5.4 ±0.18	5.1 ±0.19	13 ±0.6	247 ±11	9.7 ±0.5	9.1 ±0.5	3 ±0.9	24 ±7.2
	10	R[5]	4.8 ±0.22	4.4 ±0.20	8 ±1.0	170 ±20	10.5 ±0.9	9.8 ±0.9	1 ±0.3	6 ±2.8
Hypophysect. (2 weeks)	15	L	5.6 ±0.19	5.3 ±0.25	19 ±1.7	348 ±26	11.0 ±0.7	10.5 ±0.7	3 ±1.1	21 ±9.1
Hypophysect. (3 weeks) plus ACTH[1]	15	R	6.1 ±0.28	5.8 ±0.30	17 ±0.8	276 ±12	15.4 ±0.9	14.5 ±0.8	4 ±1.6	29 ±10.3

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
[3] μg. of ascorbic acid in the entire gland.
[4] μg. ascorbic acid/100 mg. tissue.
[5] 10 μg. ACTH injected intravenously 1 hr. prior to autopsy.
* Standard error.

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[3] Armour ACTHAR. (dosage expressed as LA-1A equivalent)

preputial and adrenal glands of the hypophysectomized rat are 60% and 37% smaller, respectively, than those of control animals. Likewise, the concentration of preputial and adrenal ascorbic acid is 58% and 44% less than that of the normal glands. In spite of the fact that the concentration of ascorbic acid is greatly reduced in these glands following hypophysectomy, they are still capable of responding to an intravenous injection of 10 μg. of ACTH by a further reduction in their ascorbic acid content (Table 1). Thus a 75% depletion of preputial and a 31% depletion of adrenal ascorbic acid was seen at one hour following the injection of ACTH.

Experiment II. The 7-day treatment with ACTH resulted in some enlargement of the preputial glands as seen by comparing their weights with those of uninjected hypophysectomized rats (Table 1). Likewise, there was a slight enlargement of the adrenal glands, although it was considerably less than was anticipated. It is clear that ACTH reverses the gradual decline in the weight of the preputial and adrenal glands seen at 2 and 3 weeks after hypophysectomy. These injections, on the other hand, had only little effect on the preputial and adrenal ascorbic acid concentrations, the values being similar to those observed in the uninjected, hypophysectomized animals.

It has been the general observation in this study that left preputial and adrenal glands are slightly heavier than right ones. This was true in the intact control group, where on a mg./100 gm. body weight basis, the left preputial gland was 18% heavier than the right and the left adrenal gland was 6% heavier than the right (Table 1). There was no significant difference, however, in the concentration of ascorbic acid in the right and left glands. In the two hypophysectomized groups, since the right glands were utilized for study after the administration of ACTH, either acutely or chronically, it is not known whether this same tendency prevails. If one assumes that left glands tend to be heavier than right ones in untreated, hypophysectomized rats, it follows that in the present study the preputial and adrenal hypertrophy produced by chronic ACTH administration is partially masked.

DISCUSSION

Following hypophysectomy there is a gradual decline both in the weight and ascorbic acid content of the preputial and adrenal glands. The results for the adrenal glands are in general agreement with the earlier findings of Tyslowitz (1943). The decline proceeds at a more rapid rate in the preputial glands where it is apparently complete at 2 weeks after hypophysectomy. It is of considerable interest that, in spite of the marked involution of the preputial and adrenal glands and their low levels of ascorbic acid, they are still sensitive to ACTH, as indicated by the further depletion of ascorbic acid following the acute administration of 10 μg. ACTH. In comparison to the adrenal gland, the preputial gland is apparently more sensitive to the ascorbic acid depleting action of ACTH.

Although the administration of ACTH for one week to animals which had been hypophysectomized 2 weeks previously was capable of reversing the gradual decline in preputial and adrenal weight, it had little effect in rebuilding the ascorbic acid content of these glands. The concentration of preputial and adrenal ascorbic acid is not significantly different from the values for the untreated, 3-week hypophysectomized animals. In a previous study of this sort Munson and Koch (1946) reported that 13 daily injections of ACTH to hypophysectomized rats raised the ascorbic acid content of their adrenal glands. Their dosage range was from 40 μg. to 1 mg. daily and they state that even when sufficient ACTH was given to increase the adrenal weight above that of unoperated controls, the adrenal ascorbic acid was still significantly less than that of the controls. It seems clear that the action of ACTH on adrenal as well as on preputial gland ascorbic acid is more complex than is indicated by the depletion phenomenon which rapidly follows the administration of a single injection. In the absence of further information it is suggested that ACTH may also influence the synthesis of ascorbic acid so that with the chronic administration of this hormone to hypophysectomized animals a new equilibrium between synthesis and utilization of ascorbic acid in these glands is reached. If this is true, it appears that at the dosage employed the activity level is well below that of the glands of intact animals, as indicated by the relatively low levels of glandular ascorbic acid. Further indication of low activity level is the relatively slight effect on the preputial and adrenal gland weight produced by the dosage of ACTH employed. Apparently the dosage of ACTH employed was near the minimally effective dosage of the preparation used.

It has been known for some time that the preputial glands of the rat are subject to androgenic stimulation (Korenchevsky, 1935). That this is not a factor in the present study is indicated by the pronounced atrophy of the testes and ventral prostate in both groups of hypophysectomized animals (Table 2). The preputial glands are also responsive to some degree to the action of estrogen and progesterone (Van der Woerd, 1938) and are currently regarded as accessory reproductive glands. Developmentally they are modified sebaceous glands and functionally they belong to the holocrine type, their secretory product being a viscous sebum. By usual histological and cytological criteria they have little in common with the adrenal cortex. It is therefore somewhat surprising that these glands show such a striking parallelism to the adrenal glands in their response to ACTH. So far as is known at present, the direct action of ACTH is limited to the adrenal cortex and the preputial glands. Long (1947) has shown that changes in ascorbic acid levels of other organs such as brain, kidney, heart,

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE BODY, TESTIS, AND PROSTATE WEIGHT OF RATS

	No. of Rats	Body weight		Left testis weight		Ventral prostate weight	
		Initial	Final	Abs.	Rel.[2]	Abs.	Rel.[2]
		gm.	gm.	mg.	mg.	mg.	mg.
Control	13	120 ±0.9*	179 ±2.9	1114 ±34	628 ±16	128 ±18	71 ±8.9
Hypophysect. (3 weeks)	14	122 ±1.7	109 ±1.8	206 ±12	191 ±11	9.5 ± 0.6	8.8 ±0.5
Hypophysect. (3 weeks) plus ACTH[1]	15	123 ±1.3	106 ±1.5	202 ±15	189 ±13	9.1 ± 0.8	8.6 ±0.8

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
* Standard error.

skeletal muscle and lymphoid organs are of an insignificant nature following a single injection of ACTH. The marked differences together with the marked similarities between the preputial and adrenal glands offer the promise that further studies of this nature will shed new light on the role of ascorbic acid in cellular processes.

SUMMARY

Hypophysectomy of male rats produces a gradual decline in both weight and ascorbic acid content of the preputial and adrenal glands. At three weeks postoperatively when the concentration of ascorbic acid is at a low level in both of these glands it can be further reduced by a single intravenous injection of ACTH. A 7-day treatment, begun two weeks postoperatively reverses the gradual decline in the weight of the preputial and adrenal glands but has little effect on their ascorbic acid content. As in intact animals, there is a close parallelism in the response of the preputial and adrenal glands to ACTH.

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ENDOCRINE INFLUENCE ON THE PLASMIN-PLASMIN INHIBITOR SYSTEM IN THE BLOOD OF RATS

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INTRODUCTION

EARLIER studies have indicated that the equilibrium of the plasmin (fibrinolysin)-plasmin inhibitor (antifibrinolysin) system in blood can be shifted under various experimental conditions (Jensen, Chamovitz, Volkringer, Gray, Gosney and Kocholaty, in press). Ungar and his associates (1951, 1951) reported that certain endocrine principles act on this system by accelerating the rate of combination of plasmin and plasmin inhibitor. The findings of the present study do not seem to substantiate this concept. Instead, the recorded data tend to show that the increased antifibrinolytic activity, observed after administration of certain hormones, is a result of an actual rise in the plasmin inhibitor level in the blood.

MATERIALS AND METHODS

Male Sprague-Dawley albino rats weighing 220–250 gm were used. The animals were allowed food and water throughout the experiment. Unless noted all injections were given subcutaneously. Controls received either no injections or a volume of solvent equal to that used in the hormone injections.

Splenectomy was performed on fasting rats anesthetized by ether. Animals regained their normal weight within the second or third post-operative day. The experimental work was carried out 7 days after splenectomy. Hypophysectomized and adrenalectomized rats, obtained from the Hormone Assay Laboratories, Inc., Chicago, Illinois, were used 10 days after operation.

The hormones were injected subcutaneously in dosages as follows:

Adrenocorticotropin, Armour Acthar, equivalent to 1.0 mg Standard LA-1-A/kg twice daily for 3 days; controls—equivalent volume of 0.9% saline.

Cortisone, Merck Cortone Acetate, 5 mg/kg daily for 3 days; controls—equivalent volume of 0.9% saline.

Thyroxine, Squibb Crystalline Thyroxine, 0.5 mg/kg daily for 4 days; controls—equivalent volume of slightly alkaline 0.9% saline.

Thyrotropin,[1] Armour Lot 2-D-3, LJW3-16-48 containing 5 Junkmann-Schoeller units per mg, 10 mg/kg daily for 5 days; controls—equivalent volume of slightly alkaline 0.9% saline.

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[1] This preparation was kindly supplied by the Armour Laboratories, Chicago, Illinois.

Blood samples, 1.8 ml, were taken by heart puncture 2 hours after the last injection, using 0.2 ml of 3.8% sodium citrate as the anticoagulant. Following centrifugation at 2800 rpm for 30 minutes at 5 to 10°C, the plasma was separated and either analyzed immediately or stored frozen until tested.

The antifibrinolytic activity of plasma was determined by measuring the residual activity of a standard amount (2 mg[2]) of bovine plasmin,[3] after incubation for 5 and 30 minutes with a 4% concentration of plasma as previously described by Westphal, Kocholaty, McDonald, King and Jensen (1950). Thrombin, salt-free, (Parke-Davis) and fibrinogen, prepared according to Ware, Guest and Seegers (1947), were used. Calculations of antifibrinolytic units (AFU) were performed as previously reported by Kocholaty, Ellis and Jensen (1952).

The plasmin inhibitor preparation used in these studies was obtained by $(NH_4)_2SO_4$ fractionation of a product prepared from bovine plasma according to Loomis, Ryder and George (1949). The plasmin inhibitor was found to be precipitated between 0.6–0.7 saturation of ammonium sulfate.

RESULTS AND DISCUSSION

When the plasmin inhibitor preparation was tested against plasmin, it was found that 340γ of this preparation were equivalent to one antifibrinolytic unit. Tested against trypsin, 60γ corresponded to one unit. This finding is in agreement with earlier observations of Christensen and MacLeod (1945) and of Lewis and Ferguson (1950) who found that the inhibition of plasmin requires a higher concentration of the serum or plasma than is necessary to inhibit trypsin of equal fibrinolytic potency.

TABLE 1. INHIBITION OF BOVINE PLASMIN BY NORMAL RAT PLASMA

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml of rat plasma ranging from 0.5–6% in the final reaction mixture. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ), the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasma concentration (%) in the reaction mixture	CDT (Seconds)*	
	5 minute incubation	30 minute incubation
0.0	123	126
0.5	129	136
1.0	135	147
1.5	145	160
2.0	153	175
2.5	166	195
3.0	182	216
3.5	203	249
4.0	234	305
4.5	275	355
5.0	314	420
5.5	382	528
6.0	455	610

* Each clot dissolution time (CDT) is the average of duplicate determinations.

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[2] This amount of plasmin dissolved the standard fibrin clot in 125 seconds at 37.5°C.

[3] Preparations of bovine plasmin were kindly supplied by Dr. E. C. Loomis of the Research Laboratories, Parke-Davis and Company, Detroit, Michigan.

The rate of combination between plasmin and its inhibitor is dependent on the time of incubation of the reactants; a 30 minute incubation produced a greater inhibition than did the 5 minute incubation, both in the case of rat plasma and of a purified preparation of bovine plasmin inhibitor (Table 1 and 2). These findings are in agreement with similar observations reported by Guest, Daly, Ware and Seegers (1948). It was found that the inhibition of trypsin by normal rat plasma after 30 minutes incubation was somewhat greater than after 5 minutes incubation, but the difference was not as pronounced as that observed with plasmin. As to be expected, the rate of combination between plasmin and its inhibitor was found to be dependent also on the temperature of the reaction mixture.

TABLE 2. INHIBITION OF BOVINE PLASMIN BY PLASMIN INHIBITOR

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml containing 20–140 of purified bovine plasmin inhibitor. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ) the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasmin inhibitor Gamma	CDT (seconds)*	
	5 minute incubation	30 minute incubation
0	126	129
20	130	138
40	131	145
60	134	154
80	137	163
100	139	166
120	142	177
140	143	193

* Each clot dissolution time (CDT) is the average of duplicate determinations.

The antifibrinolytic activity of normal rat plasma after 5 minutes incubation was 87.6 ± 7.5 units, after 30 minutes, 105.8 ± 5.8 units (Table 3). The plasmas of control rats injected either with saline or alkaline saline had similar antifibrinolytic activities. The administration of adrenocorticotropin over a 3-day period in normal rats produced a significant increase in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation, 101.4 ± 13.5 units and 119.3 ± 8.5 units, respectively, (Table 3). Thyrotropin, given for 5 days, produced an increase noted only after 5 minutes incubation. Thyroxine, administered for 4 days, produced an elevation of the antifibrinolytic activity which was evident only after 30 minutes incubation while cortisone, given for 3 days, produced no change.

Single intraperitoneal injections of adrenocorticotropin (1 to 4 mg. per rat), of adrenal cortical extract, Upjohn, (2 to 4 ml. per rat) and of thyroxine (1 mg. per rat) were found to produce no significant change in the plasmin inhibitor titer 15 to 90 minutes after administration.

The antifibrinolytic activity of plasma 10 days after removal of the hypophysis or of the adrenals was found to be greatly reduced (Table 3). Splenectomy did not alter the plasmin inhibitor level of plasma (Table 3). In splenectomized rats, adrenocorticotropin administration (same procedure as in normal animals) did not produce any change in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation (Table 3). Apparently, the influence of the adrenals on the plasmin inhibitor titer is mediated through the spleen. In this connection it may be pointed out that Macfarlane and Biggs (1948) have observed that among all the organs, extracts of the spleen had the highest antifibrinolytic activity. On the other hand, administration of thyroxine (same procedure as in normal animals) to the splenectomized rat produced a significant elevation of the antifibrinolytic titer as measured after 30 minutes incubation (Table 3). Apparently, more than one mechanism is involved in the control of the plasmin inhibitor level in blood.

TABLE 3. EFFECT OF REMOVAL OF ENDOCRINE ORGANS AND OF VARIOUS HORMONES ON THE ANTIFIBRINOLYTIC ACTIVITY OF RAT PLASMA

Treatment	No. of animals	Incubation time					
		5 Minutes			30 Minutes		
		CDT[1] Avg. Sec.	AFU[2]/ml plasma Avg. ±S.D.	t[3]	CDT Avg. Sec.	AFU/ml plasma Avg. ±S.D.	t
Normal Animals							
No treatment	13	234	87.6 ± 7.5		284	105.8 ± 5.8	
Saline	10	238	88.5 ± 9.3		295	108.8 ± 7.6	
Alkaline Saline	10	234	87.6 ± 8.3		291	107.9 ± 9.0	
ACTH‡	15	274	101.4 ± 13.5	2.8†	339	119.3 ± 8.5	3.2‡
Cortisone	15	240	88.9 ± 12.8	0.9	311	112.7 ± 9.7	1.1
Thyroxine	15	231	85.3 ± 8.6	0.4	318	114.9 ± 6.2	2.2*
TSH	15	255	95.6 ± 8.3	2.6†	311	113.0 ± 7.7	1.5
Hypophysectomy	4				202	60[4]	
Adrenalectomy	4				230	76[4]	
Splenectomy[5]							
Saline Controls	3	239	89.0		284	107.0	
ACTH	9	254	94.3 ± 12.7	1.1	298	109.7 ± 7.7	0.3
Thyroxine	9	242	90.9 ± 8.1	1.1	327	117.1 ± 6.9	2.5*

[1] Clot dissolution time.

[2] Antifibrinolytic units.

[3] Comparisons are made with the injected controls. p values: * = <0.05, † = <0.02, ‡ = <0.01.

[4] Tested against standard trypsin after 30 minutes incubation only. The normal value with trypin—104 AFU/ml of plasma.

[5] Previous studies showed no changes in the antifibrinolytic titer after splenectomy.

In general, these findings are in agreement with the observations of Ungar and coworkers (1951) namely, an increased antifibrinolytic activity after the administration of adrenocorticotropin to normal animals and no alteration in the plasmin inhibitor titer when administered to splenectomized animals. However, the results presented do not seem to substantiate the concept suggested by these workers that adrenocorticotropin affects the antifibrinolytic activity of normal plasma by accelerating the rate of combination between plasmin and plasmin inhibitor. Instead, the recorded data tend to show that the observed increased antifibrinolytic activity is a result of an actual rise in the plasmin inhibitor level in the blood.[4]

SUMMARY

The degree of combination between plasmin and plasmin inhibitor was found to depend on the incubation time and temperature of the reactants.

Administration of adrenocorticotropin produced a significant increase in the plasmin inhibitor titer of blood. The injection of thyrotropin or thyroxine caused elevations of the plasmin inhibitor in blood. Cortisone produced no change. The plasmin inhibitor titer of rat plasma 10 days after removal of the hypophysis or of the adrenals, was found to be greatly reduced. Splenectomy did not alter the plasmin inhibitor level of plasma. In splenectomized rats adrenocorticotropin administration did not produce any change in the plasmin inhibitor titer of the blood. On the other hand, administration of thyroxine to splenectomized rats was found to produce an elevation of the titer.

The significance of these findings has been discussed.

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[4] It may be pointed out that the observed increase in antiplasmin activity can also be caused by a decrease in the plasmin of the blood. The test, as employed, does not differentiate between fibrinolytic and antifibrinolytic activity.

NOTES AND COMMENTS

THE INFLUENCE OF CORTISONE ON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID*

Several groups of workers have found a depression of thyroid I^{131} uptake in cortisone-treated rats (Money *et al.*, 1951, Verzár and Vidovič, 1951, Perry, 1951, Albert *et al.*, 1952). An inhibition of the pituitary thyrotrophic mechanism was ruled out as the cause of this phenomenon by the findings that the rate of release of radioiodine from the thyroid is not reduced by cortisone (Perry, 1951, Albert *et al.*, 1952) and that the morphology of the thyroid in cortisone-injected rats suggests a compensatory hypersecretion rather than a diminished output of thyrotrophin (TSH) (Halmi and Barker, 1952). Albert *et al.* (1952), in a trenchant analysis of the cortisone-induced changes in thyroid function, pointed out that reduced I^{131} uptake may be the result of various disturbances, such as an *increased renal elimination of iodide, an augmentation of the iodide space* or an inhibition of the concentration of iodide by the thyroid. One might add to this list an interference with the organic binding of iodine in the thyroid.

The present study was undertaken to ascertain the effect of large doses of cortisone on the ability of the thyroid to concentrate iodide. Since Woodbury *et al.* (1951) reported that exogenous TSH is less effective in raising the thyroidal I^{131} uptake of hypophysectomized rats if cortisone is administered simultaneously, we also investigated the influence of cortisone on the thyroid iodide trap of hypophysectomized rats receiving TSH. Finally we examined the effect of cortisone on the rise in the thyroid: serum iodide ratio which follows the administration of goitrogens (Vanderlaan and Vanderlana, 1947).

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain were used in this study. In the first experiment 10 rats were injected with daily doses of 8 mg of cortisone[1] for 10 days. Ten uninjected control rats were pair-fed. In the second experiment hypophysectomized rats (100–150 gm.) were obtained 8 days after operation from the Hormone Assay Laboratories, Chicago and divided into 3 groups. The first group received no treatment, animals in the second group were injected with 1 mg. of TSH (USP Thyrotropin Reference Substance) daily for 10 days, while to those of the third group 1 mg. of TSH and 5 mg. of cortisone were administered daily over a period of 10 days. In the third experiment 10 rats received 10 daily injections of 10 mg. of propylthiouracil in an alkaline solution, while 10 pair-fed rats were given 10 mg. of cortisone daily for 10 days in addition to the same amount of propylthiouracil. All unoperated rats received powdered Rockland chow, while the hypophysectomized animals were given the same diet in pellet form and supplemented with horse meat and oranges.

The iodide concentrating ability of the thyroids was determined by the method of Vanderlaan and Greer (1950). All rats were injected subcutaneously with 5 mg. of propylthiouracil in an alkaline solution. Forty-five minutes later they received an injection of 5 μc. of carrier-free radioiodide.[2] One or two hours following the latter injection the rats were anes-

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[1] Cortisone (Cortone acetate Merck) was generously supplied by Dr. E. Alpert, Merck and Co., Rahway, N. J.

[2] Radioiodide was obtained through the courtesy of Dr. T. C. Evans, Radiation Research Laboratory, State University of Iowa.

thetized with ether and blood was withdrawn from the abdominal aorta. The thyroids were dissected out, weighed on a torsion balance and squashed on a piece of filter paper, which was placed on an aluminum planchet and covered with scotch tape. One tenth of a ml. of serum was pipetted onto a piece of filter paper of the same size, which then was also fixed to a planchet with scotch tape. The radioactivity of the thyroids and the blood samples was determined by means of a thin mica-window Geiger-Müller counter. Since the propylthiouracil almost completely prevents the organic binding of the subsequently administered I^{131} (Vanderlaan and Vanderlaan, 1947), the radioactivity of the thyroid can be regarded as the measure of radioiodide collection by the gland. The thyroid:serum iodide ratio is expressed as the quotient of the respective activities of 100 mg. of thyroid tissue and 0.1 ml. of serum.

RESULTS

Table 1 shows that the thyroid:serum iodide ratio in rats treated with 8 mg. of cortisone daily for 10 days was somewhat higher than that of pair-fed controls. Table 2 summarizes the observations on groups of hypophysectomized rats which received a) no treatment, b) 1 mg. of TSH daily for 10 days, and c) 1 mg. of TSH and 5 mg. of cortisone daily for 10 days. The low thyroid:serum iodide ratio found in the untreated hypophysectomized rats is in agreement with the findings of Greer (1949). TSH markedly elevated the thyroid:serum iodide gradient and cortisone did not exert any significant influence on this response to TSH. The results of the third experiment, which are shown in Table 3,

TABLE 1. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS GIVEN 8 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF THEIR PAIR-FED CONTROLS

Group	No. of rats	Average: Starting weight, gm.	Average: Final weight, gm.	Thyroid: serum iodide ratio (mean ±st. error)	
Cortisone	10	194	153	27.9 ±2.6	P=0.02
Control	10	210	213	20.8 ±0.4	

TABLE 2. THYROIDAL IODIDE CONCENTRATING ABILITY OF GROUPS OF HYPOPHYSECTOMIZED RATS RECEIVING NO TREATMENT, 1 MG. OF TSH DAILY FOR 10 DAYS OR 1 MG. OF TSH+5 MG. OF CORTISONE DAILY FOR 10 DAYS

	No treatment	TSH only	TSH+cortisone
No. of rats	14	13	13
Thyroid: serum iodide ratio (mean ±st. error)	5.0 ±0.7	32.9 ±2.6	30.2 ±2.6
		P=0.4	

TABLE 3. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS RECEIVING 10 MG. OF PROPYLTHIOURACIL+10 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF PAIR-FED CONTROLS GIVEN 10 MG. OF PROPYLTHIOURACIL DAILY FOR 10 DAYS

Group	No. of rats	Average: Starting weight, gm.	Average: Final weight, gm.	Thyroid: serum iodide ratio (mean ±st. error)	
Propylthiouracil+cortisone	10	195	152	92.7 ±15.7	P=0.7
Propylthiouracil only	10	199	193	101.8 ± 6.4	

indicate that the simultaneous administration of 10 mg. of cortisone and of 10 mg. of propylthiouracil daily failed to prevent the rise in the thyroid:serum iodide ratio which was elicited by 10 daily doses of 10 mg. of the latter drug alone.

DISCUSSION

The results presented in this paper indicate a) that the defective thyroid I^{131} uptake observed by others in cortisone-treated rats is not likely to have been the sequel of an interference of this steroid with the ability of the thyroid to concentrate iodide, b) that the partial inhibition exerted by cortisone upon the TSH-induced rise in thyroid I^{131} uptake of hypophysectomized rats (Woodbury *et al.*, 1951) is equally unlikely to have been the result of interference with the iodide trapping mechanism, and c) that, indeed, a striking elevation of the thyroid: serum iodide ratio in response to a goitrogen is compatible with the simultaneous injections of large amounts of cortisone. The fact that cortisone-treated rats had an apparently slightly higher thyroid:serum iodide ratio than that of their pair-fed controls is in agreement with the concept that this steroid is not a TSH inhibitor, since the thyroid:serum iodide gradient drops if TSH does not act on the thyroid. Furthermore, it harmonizes with histological findings indicating an increased thyrotrophic stimulation of the thyroids of cortisone-treated rats (Halmi and Barker, 1952). Since both the pituitary thyrotrophic mechanism and the thyroid iodide trap thus appear to be ruled out as points of attack of cortisone, the following hypotheses remain to be verified or refuted: a) cortisone affects the extrathyroidal metabolism of iodide (renal excretion, iodide space), b) it partially inhibits a phase of the organic binding of iodine in the thyroid.

SUMMARY

Cortisone did not materially affect the iodide trapping ability of the thyroids of normal rats, of hypophysectomized rats given thyrotrophin, or of rats chronically treated with propylthiouracil.

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THE USE OF COLLODION ULTRAFILTRATES OF HUMAN URINE FOR STEROID DETERMINATIONS

INTRODUCTION

The practicability of determining the content of more than one hormone in a single sample of human urine is limited, essentially, by the concentration of the desired hormones in the urine. Thus, the gonadotrophins, pregnanediol and the adrenal cortical steroids are usually present in such small amounts, particularly in non-pregnant subjects, that quantities of urine varying from about $\frac{1}{5}$ of a day's output to as much as a 48 hour output, or more, may be required for the determination of only one of these. It is often desirable, however, from the standpoint of the patient's convenience, as well as the research objective, to know the excretion level of the gonadotrophins and of a steroid hormone or its metabolites during a particularly limited period of time, such as 24 or 12 hours. Our use of the Gorbman (1945) method for extracting gonadotrophins by ultrafiltration through a collodion membrane suggested that a simple solution to this problem might be at hand if the ultrafiltrate could be used for the steroid assays. This has been tested by determination of the 17-ketosteroid and pregnanediol content of several urines before and after filtration through collodion. We have found that, with certain reservations, the technique is a practical one, and, to our knowledge, has not been reported before.

METHODS AND MATERIALS

A total of 39 complete 24 hour urines were studied for total 17-ketosteroid, and 26 urines were studied for pregnanediol content before and after ultrafiltration. The urines were collected from a group of young healthy female inmates of a home for the feeble minded. All urines were kept refrigerated, with thymol as the only preservative, until used.

Gonadotrophins were extracted on aliquots of each urine, as described by Gorbman (1945). In the pregnanediol series, a single aliquot of urine, equal to $\frac{1}{5}$ of the day's output, was first removed, the remaining $\frac{4}{5}$ of the day's urine ultrafiltered, and pregnanediol determined on a single $\frac{1}{5}$ aliquot of the ultrafiltrate, and on the aliquot of unfiltered urine. For the 17-ketosteroid series, a 10.0 cc. volume of unfiltered urine was first removed, the remainder of the urine ultrafiltered, and the 17-ketosteroid determinations carried out on a 10.0 cc. volume of the ultrafiltrate and the 10.0 cc. aliquot of unfiltered urine. The method of Sommerville, Gough and Marrian (1948) was used for pregnanediol, and that of Drekter, *et al.* (1947) for 17-ketosteroid analyses.

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TABLE 1. TOTAL 17 KETOSTEROID CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values are presented in three groups: (A) urines containing less than 10.0 mg./24 hours; (B) urines containing between 10.1 and 15.0 mg./24 hours; and (C) urines containing more than 15.0 mg./24 hours. The values shown in the first two columns of each group are mg. of 17-ketosteroid per 24 hours. The group means for the percentage change from the value of the urine before filtration are derived from the difference between the before and after filtration group means, rather than from the sum of the individual percentages. There is a slight discrepancy between these two values only in the case of the first group of urines (less than 10.0 mg./24 hours).

A. Less than 10.0 mg. per 24 hours			B. 10.1 to 15.0 mg. per 24 hours			C. More than 15.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
4.9	5.8	+18	10.1	7.8	−23	15.1	13.9	−7.9
5.0	6.3	+26	10.8	8.0	−26	15.8	14.6	−7.6
6.3	5.2	−17	10.9	8.3	−24	16.0	16.0	0
6.4	9.5	+48	11.2	9.7	−13	16.0	16.0	0
6.6	8.0	+21	11.4	9.5	−17	16.6	15.3	−7.8
6.9	6.0	−13	12.6	12.6	0	17.3	14.7	−15
7.2	7.9	+9.7	13.1	13.1	0	17.5	17.0	−2.9
7.3	4.8	−34	13.2	10.5	−20	20.4	19.9	−2.4
9.2	7.0	−24	13.4	15.3	+14	20.9	14.4	−31
9.4	11.4	+21	15.0	15.0	0	21.6	19.6	−9.3
9.5	8.0	−16				21.9	21.9	0
9.8	7.2	−27				22.0	21.2	−3.6
9.9	6.6	−33				22.6	22.6	0
						25.5	25.2	−1.2
						26.5	21.4	−19
						48.6	50.3	+3.5
Group means:								
7.6	7.2	−5.3	12.2	11.0	−9.9	21.5	20.2	−6.1
Mean of % change, regardless of sign:		23.7			13.7			6.9

* From the value of the urine before filtration.

RESULTS

17-ketosteroids. The 17-ketosteroid values, in terms of mgs./24 hours, are shown in Table I. The values are arranged in three groups, according to the quantity of steroid excreted per day, in order to show its relation to the percentage change (expressed in all cases in relation to the amount present in the urine before filtration) following filtration. There was a very obvious inverse relation between the daily level of 17-ketosteroid excretion and the amount of variation in the values of the percentage change following ultrafiltration. This is shown by the decrease in the value of the mean of the percentage change, *calculated without regard to the sign of this change* (shown at the bottom of each group of values). The true means showed little difference between the three groups of urines, and the fact that most of the minus changes were found in the intermediate and high content urines is an indication of a probable slight but real loss of steroid as a result of filtration.

Pregnanediol. Not as many urines were examined for pregnanediol content, and the values, though extending over as wide a relative range as did the 17-ketosteroids, did not compare with the latter as far as absolute amounts are concerned. Nevertheless, very much the same sort of relationship was seen (Table 2), and what has been said about the

TABLE 2. PREGNANEDIOL CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values presented are in two groups: (A) urines containing less than 1.0 mg./24 hours; and (B) urines containing more than 1.0 mg./24 hours. As in Table 1, the values in the first two columns of each group are mg. of pregnanediol per 24 hours, and the group means for the per cent change are derived from the difference between the before and after filtration group means.

A. Less than 1.0 mg. per 24 hours			B. More than 1.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
0.17	0.25	+47	1.17	0.90	−23
0.19	0.09	−52	1.35	0.44	−67
0.20	0.20	0	1.37	1.19	−13
0.25	0.20	−20	1.38	1.47	+6.5
0.26	0.28	+7.7	1.62	1.41	−13
0.26	0.22	−15	1.70	1.48	−13
0.28	0.23	−18	1.70	1.77	+4.1
0.28	0.39	+39	1.77	1.55	−12
0.36	0.58	+61	1.89	1.68	−11
0.40	0.25	−37	1.99	1.90	−4.5
0.74	0.55	−26	2.07	2.34	+13
0.86	0.13	−85	2.42	2.23	−7.9
0.96	0.21	−78	4.90	7.50	+53
Group means:					
0.40	0.27	−32	1.95	1.97	−1.03
Mean of % change, regardless of sign		37%			19%

* From the value of the urine before filtration.

size of the change following filtration and the amount of 17-ketosteroid in the urine, applies in general as well to the pregnanediol series.

DISCUSSION

When one considers that the two values compared in this study were obtained from single aliquots of urine, the amount of variation between them is not surprising and should not be disturbing. For clinical purposes there can hardly be any question of the value of using the ultrafiltrate of urine for a steroid determination, since the alternative of using separate 24 hour collections will be associated with variations at least as large as those resulting from the passage through collodion. In addition, the importance of such determinations for clinical purposes is not obviated by a loss or gain from the real value of roughly 20%. However, the difference from the true value as a result of filtration may be serious in certain areas of research where relatively small differences in output are anticipated, but this is to some extent minimized by the fact that the percentage change is more frequently a large one almost only when one is dealing with relatively small concentrations of steroid. If this is recognized, and data interpreted accordingly, there is no reason why the method cannot be used, especially if the determinations are made on at least duplicate aliquots of the ultrafiltrate. It has thus been of inestimable help in allowing us to perform a single experiment to determine simultaneously the effect of progesterone on gonadotrophin output, and the conversion of progesterone to pregnanediol in relation to the phases of the menstrual cycle.

SUMMARY

The 17-ketosteroid and pregnanediol content of human urine after ultrafiltration through collodion is shown to vary from the content of these steroids in the unfiltered urine within limits small enough to allow the ultrafiltrate to be used for the determination of excretion levels of these substances.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.

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COMPARATIVE EFFECT OF ACTH, CORTISONE AND DCA ON RENAL FUNCTION, ELECTROLYTE EXCRETION AND WATER EXCHANGE IN NORMAL DOGS

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THE inadequacies of our knowledge of the role of adrenocortical hormones in the regulation of electrolyte and water metabolism are becoming increasingly apparent (Gaunt, *et al.*, 1949; Ingle, 1950; Sayers, 1950). Extensive and numerous observations on the action of DCA have been reported (Ragan, *et al.*, 1940; Ferrebee, *et al.*, 1941; Mulinos, *et al.*, 1941; Zierler and Lilienthal, 1948; Gaudino and Levitt, 1949; Davis, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952), but data on the effect of 11,17-oxysteroid hormones in the dog are meager (Thorn, *et al.*, 1941; Davis, *et al.*, 1951; Roberts and Pitts, 1952). The latter studies were with cortisone and observations with ACTH are necessary before adequate evaluation of the relation of the ACTH-adrenocortical system to salt metabolism can be made. An important consideration which has been neglected in most studies with ACTH and cortisone in the dog and rat is the role of glomerular filtration rate in the regulation of salt and water excretion. As demonstrated in this report, data on alterations in the rate of glomerular filtration are essential for satisfactory evaluation of electrolyte excretion during hormone administration.

The present study was undertaken to help clarify the relation of adrenocortical hormones to salt and water metabolism. The salt retaining effects of ACTH, cortisone, and DCA have been compared and the relation of glomerular filtration rate to changes in electrolyte excretion and water

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exchange has been studied. Since the response to adrenocortical hormones differs among species, the results in the dog have been compared with those obtained previously in man (Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952) and rat (Rice and Richter, 1943; Ingle, *et al.*, 1946; Bergner and Deane, 1948; Boss, *et al.*, 1950).

MATERIALS AND METHODS

ACTH, cortisone, and DCA were administered to four normal female mongrel dogs. After a 2 to 3 week control period, these hormones were given successively for intervals of 5 to 31 days; a recovery period was allowed following each hormone injection period. A saline solution of ACTH[1] was injected intramuscularly every six hours whereas saline suspensions of cortisone acetate and DCA in oil were given intramuscularly once daily. Approximate doses of 2.1 and 6.3 mgm./kg./day of ACTH and 2.1 and 4.2 mgm./kg./day of cortisone were employed for dogs 1 and 2, but dogs 3 and 4 received the larger dose only (Figures 1–4). All four animals received approximately 1.0 mgm./kg./day of DCA. Two levels of sodium (Na) intake (0.5 and 3.3 m.Eq./kg./day) were given during cortisone administration to dog 3 and during injection of all three hormones in dog 4; otherwise, observations were made during the higher level of Na intake.

Glomerular filtration rate (GFR) and effective renal plasma flow (RPF) were determined in the postabsorptive state by measuring the renal clearances of creatinine (C_{CR}) and para-aminohippurate (C_{PAH}) respectively. Renal clearances were measured from 3 to 5 hours after hormone administration. During the renal function studies, blood was drawn for determination of plasma electrolytes and arterial plasma carbon dioxide content. T-1824 dye space was measured and circulating eosinophils enumerated. Femoral arterial blood pressure was determined following the renal clearance procedure.

The dogs were kept in metabolic cages except during experimental procedures. Sodium, potassium (K), chloride (Cl), nitrogen (N), and water balance studies were made according to methods reported previously (Davis and coworkers, 1952). A synthetic diet consisting of 80 Cal./kg./day was force fed so that intake was constant except during vomiting or parenteral administration of additional electrolytes. Water was allowed *ad libitum.*

The experimental procedures and chemical methods have been described elsewhere (Davis, *et al.*, 1952) with these exceptions: (1) plasma Cl in dogs 3 and 4 and the Cl content of food and feces were determined by a modified Volhard technique, (2) the carbon dioxide content of arterial plasma was measured manometrically by the method of Van Slyke and Neill (1924), and (3) for the eosinophil counts, a mixture of propylene glycol and phloxine was used as a diluent (Speirs and Meyer, 1949).

RESULTS

Alterations in renal hemodynamic function were qualitatively similar for all three hormones (Figs. 1–3). The response consisted of an increase in GFR and RPF while filtration fraction (FF) fell. Renal blood flow was also elevated since hematocrit remained at the control level. The magnitude of the effect varied considerably among animals as well as with the hormone administered. The response was greatest with cortisone, least with DCA, and moderate with ACTH. The most marked elevations occurred in dogs 1 (Fig. 1) and 3 in which renal function increased after a single injection of each hormone. The least response resulted in dog 2 (Fig. 2) in which renal function was not detectably altered during ACTH

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[1] The ACTH was the ACTHAR product of Armour Laboratories; each unit was equivalent to 1 mgm. of the lyophilized standard LA-1-A.

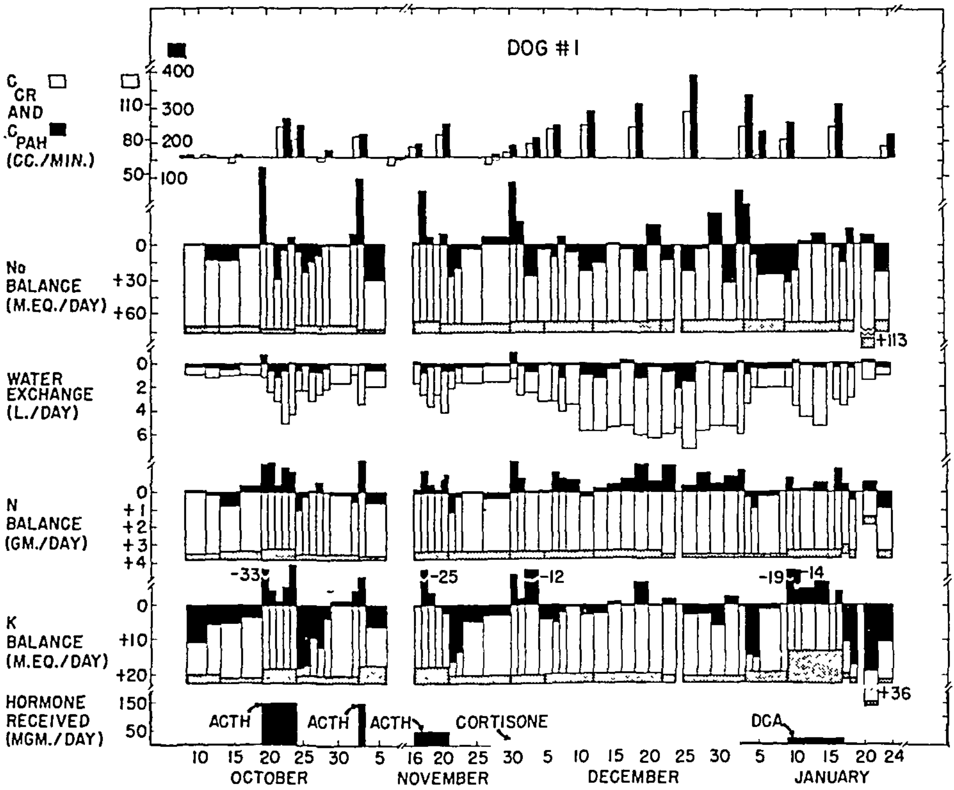

FIG. 1. The effect of ACTH, cortisone, and DCA on renal function and metabolic balances in a 24 kg. dog. Values for C_{CR} and C_{PAH} were plotted as open and solid columns respectively from a horizontal line which represents the control level. Each value is the average of 3 postabsorptive clearance periods. The convention for the balances is as follows: (1) intake was plotted downward from the zero line, (2) output was plotted upward from intake line, (3) solid black areas represent total balances, and (4) the cross-hatched areas show fecal excretion. Consequently, solid black area above the zero line indicates negative balance and black area below the zero line represents positive balance.

administration, and a slight elevation occurred only after several days of cortisone or DCA. In dog 4, GFR and RPF were increased after a single injection of ACTH and cortisone; no apparent difference was observed between the effect on low and high Na intake. DCA failed to increase renal function in dog 4 even after prolonged administration on a high Na intake.

Another striking effect of these hormones was the increase in water exchange. The typical response (3 of 4 dogs) to ACTH and cortisone was an increase in water turnover from about ½ to approximately 2 L./day (Figs. 2 and 3) but in dog 1 water exchange reached 6 to 7 L./day (Fig. 1). In this dog, the magnitude of water turnover showed a high positive correlation with the level of GFR for all three hormones and the onset of polyuria preceded the development of polydipsia. The response to DCA was less variable among the animals and a diabetes insipidus-like state occurred in all cases on a high salt diet (Figs. 1–3). The level of water exchange was independent of Na intake during cortisone administration (cases 3 and 4; see Fig. 3), but DCA had no influence on water turnover on a low Na intake (dog 4) (Fig. 3). The effect of Na intake on water exchange during ACTH administration was equivocal (Fig. 3).

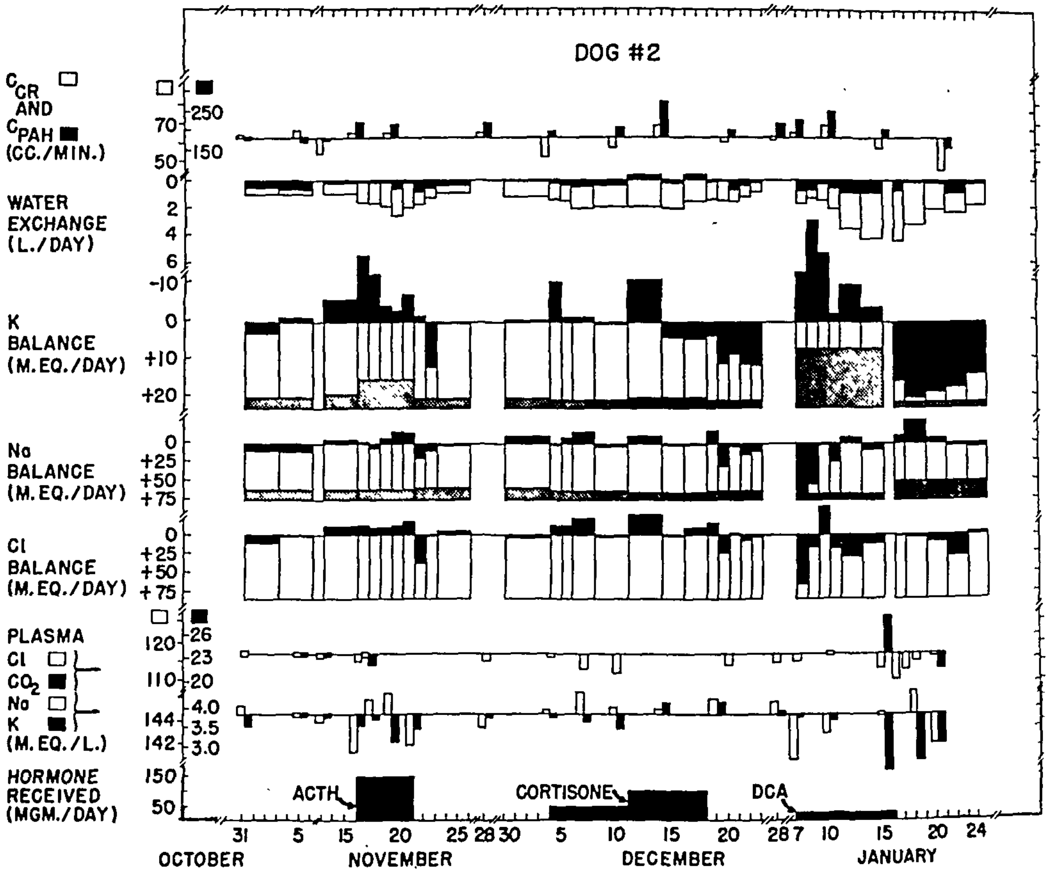

FIG. 2. Alterations in renal function, water exchange, electrolyte balances, and plasma electrolytes during ACTH, cortisone, and DCA administration to a 24 kg. dog. The horizontal line from which values for plasma electrolytes are plotted represents the average control value. See Figure 1 for the convention for the balance plots and other symbols.

The first 1 or 2 days of ACTH and cortisone injections were accompanied by salt loss in dogs 1, 3, and 4; GFR was elevated initially in these three dogs but not in dog 2 (Figs. 1–3). Salt retention resulted during the corresponding period on DCA. Thereafter, Na and Cl balance was maintained on ACTH, cortisone, and DCA although wide daily fluctuations in Na and Cl excretion occurred during prolonged cortisone administration (Fig. 1). Fecal Na excretion was unaffected by ACTH and cortisone but DCA effected a slight depression which was followed by an elevation during the recovery period in cases 2 (Fig. 2) and 3.

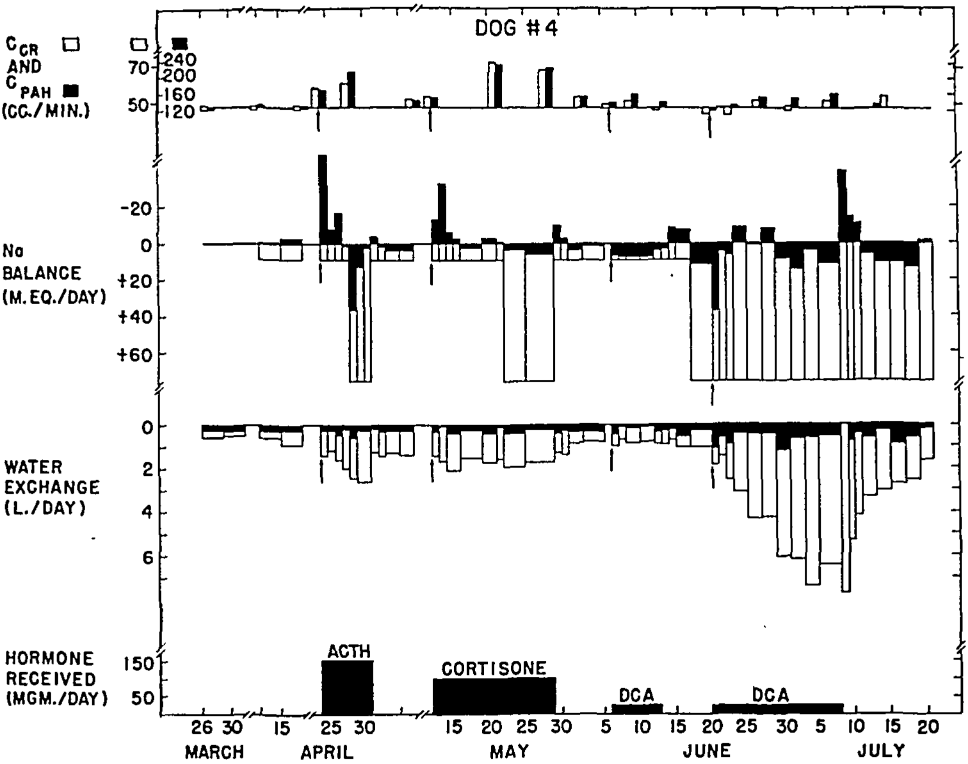

FIG. 3. Renal function, Na balance, and water exchange in a 22 kg. dog given ACTH, cortisone, and DCA during a low and a high Na intake. For a description of symbols and convention for the balances, see Figure 1.

Urinary K excretion increased initially with all three hormones during high salt intake; later, the influence of cortisone became negligible and K loss during ACTH and DCA diminished (Figs. 1, 2, and 4). In dog 4, the initial loss of K also occurred on a low salt diet with ACTH, but cortisone and DCA failed to produce a negative K balance unless salt intake was high. The greatest increase in fecal K output accompanied DCA but an elevation also occurred with ACTH except in case 3; cortisone was without effect.

Negative K balance was most marked with DCA which resulted in a metabolic alkalosis in all instances except during low salt intake in dog 4 (Figs. 1, 2, and 4). After 8 to 10 days of DCA injections, dogs 1 and 2 exhibited signs of K deficiency including generalized muscle weakness, nystagmus, lassitude, abdominal distention, and vomiting. In contrast, dog 4 was given DCA for 18 days without clinical evidence of K deficiency although plasma K dropped to 1.9 m.Eq./L. (Fig. 4). Plasma K and water exchange became progressively more abnormal as long as DCA was given, but in dog 4 plasma CO_2 reached a maximum after 11 days and remained unchanged until DCA was discontinued. Hypochloremia occurred during the DCA alkalosis in dogs 1–3 (see Fig. 2) but not in dog 4. Plasma Na was elevated during DCA administration in all but dog 2. ACTH and cortisone did not detectably alter plasma electrolytes.

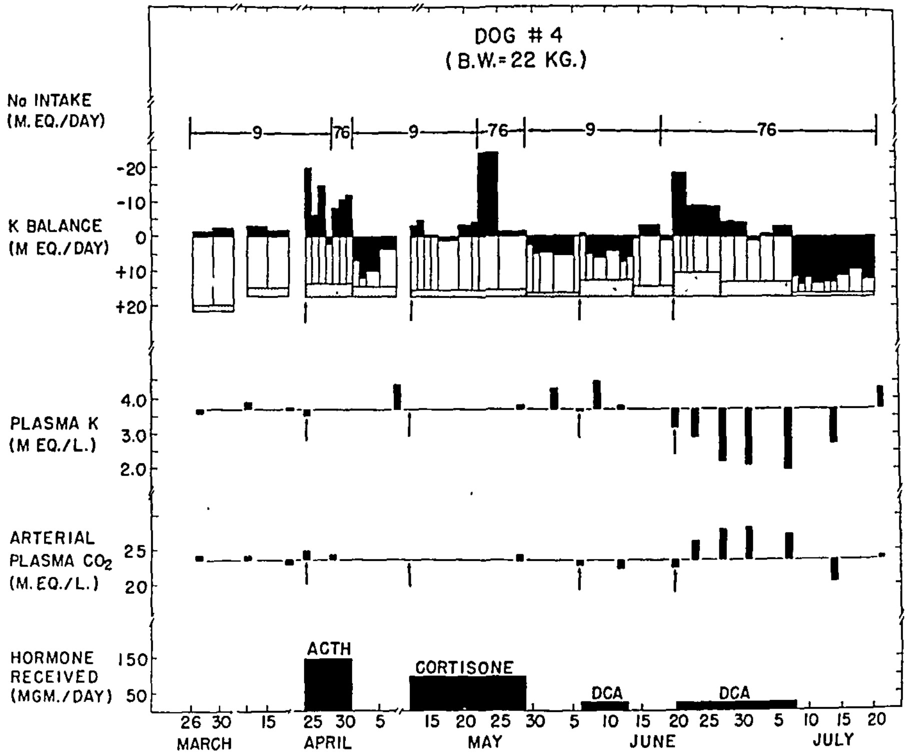

FIG. 4. Potassium balance, plasma K, and arterial plasma CO_2 during ACTH, cortisone, and DCA administration on a low and a high Na diet. Values for plasma K and arterial plasma CO_2 were plotted from a horizontal line which represents the control level. The convention for K balance has been given with Figure 1.

A negative nitrogen balance occurred throughout ACTH and cortisone administration but the only consistent effect of DCA was increased N excretion on the first day of injection. Circulating eosinophils decreased or disappeared during injection of ACTH and cortisone in dogs 1, 2, and 4; in dog 3, repeated studies before and after ACTH administration failed to reveal a decline in eosinophils and the effect of cortisone was less than in the other cases. T-1824 dye space, hematocrit, plasma proteins, and femoral arterial blood pressure were not detectably changed. Heart rate increased only during the alkalosis with DCA.

DISCUSSION

The typical response in the renal circulation to ACTH, cortisone, and DCA was an increase in the flow of blood through the kidney. Renal arteriolar dilatation was indicated by the finding of elevated renal blood flow while arterial pressure remained at the control level. The response in renal function was observed from 3 to 5 hours after a single injection for all three hormones in dogs 1 and 3. This observation may indicate that the initial rise in renal function was effected by an action on the renal arterioles. The sustained elevation in renal function may also have resulted in part from a direct renal effect. On the basis of available data, the response to ACTH and cortisone cannot be related to expansion of the extracellular fluid volume as has been suggested for DCA by Gaudino and Levitt (1949) since the initial effect of ACTH and cortisone was a natriuresis and balances for salt and water were never positive. However, the possibility that ACTH and cortisone can effect a shift in fluid from the intracellular to the extracellular compartment as reported by Levitt and Bader (1951) has not been excluded.

The pattern of renal functional changes in the dog differs slightly from that found for man (Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951). In both species, GFR and RPF may be elevated by ACTH and cortisone but in man FF is usually increased whereas in the dog it is reduced. In the rat, no significant alteration in GFR was noted during injection of cortisone (Cotlove, unpublished data), but DCA has been reported to decrease GFR slightly (Boss, *et al.*, 1950); these studies were not accompanied by measurements of RPF.

Water exchange was increased by all three hormones but the phenomenon produced by DCA appeared to differ from that with ACTH and cortisone both quantitatively and qualitatively. A diabetes insipidus-like state occurred with DCA only, except in dog 1 in which water exchange was markedly elevated with both ACTH and cortisone. In this animal the high positive correlation between the level of GFR and water exchange may indicate that GFR played a role in the development of the diabetes insipidus-like state. Also, in dog 1 the primary polyuria during both ACTH and cortisone administration may be a reflection of the initial elevation in GFR. During injection of cortisone, water turnover was unaffected by changing the level of Na intake, whereas, as shown previously by Mulinos and associates (*1941*) for the dog and by Rice and Richter (1943) for the rat, the level of water exchange during DCA is a function of salt intake. In man, the level of water turnover is uninfluenced by ACTH, cortisone, or DCA (Prunty, *et al.*, 1948; Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Sprague, *et al.*, 1950; Alexander, *et al.*, 1951; Sjögren, 1952).

Distortion of the plasma electrolyte pattern and development of alkalosis which frequently results during ACTH or cortisone administration in man (Prunty, *et al.*, 1948; Sprague, *et al.*, 1950; Sjögren, 1952) failed to occur in the dog. The duration of ACTH administration may have been too short to result in alkalosis, but the dose and duration of cortisone injection were sufficient to demonstrate a negative result. The negative K balance which occurred during ACTH and cortisone was probably a reflection of increased tissue catabolism since a concomitant loss of N resulted. The data indicate that the marked K depletion which accompanied DCA resulted from a different mechanism. It seems unlikely that K loss and alkalosis during DCA administration were a passive consequence of Na retention as has been proposed by Seldin and coworkers (1951) since Na retention lasted from 1 to 2 days only whereas a negative K balance was present throughout DCA administration. A more plausible explanation is that K depletion was the primary event. Under this circumstance, it has been suggested by Cooke, *et al.* (1952), that hydrogen ions move intracellularly with Na in exchange for K and result in alkalosis. Also, K ions may be less available for distal tubular exchange for Na than normally so that sufficient hydrogen ion secretion by the kidney occurs to prevent disappearance of the alkalosis (Berliner, Kennedy, and Orloff, 1951). Failure of alkalosis to develop with DCA during low salt intake has been reported previously by Seldin and associates (1951) and is apparently related to inadequate anion excretion. Excessive loss of K by the fecal route may indicate a direct influence of adrenocortical hormones on the intestinal mucosal transport of electrolytes (Dennis and Wood, 1940).

The effect of adrenocortical hormones on salt excretion also varies among species. An initial natriuretic and chloruretic action has been reported during administration of ACTH to man (Forsham, *et al.*, 1948) and rat (Ingle and associates, 1946; Bergner and Deane, 1948), but the salt retention which follows the initial period in man has not been observed in the rat. In the present study in the dog, both ACTH and cortisone produced an initial salt loss and, like the effect in the rat, Na retention did not occur later. It has been suggested that the negative balances for Na and Cl in man resulted from a temporary increase in 11,17-oxysteroid secretion before the effect of DCA-like hormones became manifest (Forsham, *et al.*, 1948). In the dog, it seems more likely that salt loss resulted from elevated GFR; the natriuresis occurred only in the three dogs with an initially increased GFR and not in the other animal in which GFR remained at the control level.

In contrast to the initial effect of ACTH and cortisone on salt excretion in the dog, DCA resulted in Na retention in spite of elevated GFR in two of the four dogs. This finding indicates that the Na retaining effect of DCA is greater than that of cortisone or adrenocortical hormones released by ACTH at the dosage levels employed. The effect of DCA on electrolyte metabolism appears to be similar in dog and man except that negative Na and Cl balances have been reported in man following prolonged (34–60 days) administration (Sjögren, 1952).

After the initial response, salt balance was maintained during administration of all three hormones. GFR was frequently elevated so that increased tubular reabsorption of Na and Cl can be inferred in these instances. It is not certain from these data whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules or if salt balance was achieved by another mechanism. A salt retaining effect of cortisone in the normal dog was likewise equivocal. In the adrenalectomized dog, however, cortisone appears to have a direct renal tubular action to conserve Na. Roberts and Pitts (1952) found a marked reduction in the renal excretion of Na while GFR remained at the control level. Also, adrenalectomized dogs have been maintained in excellent health for several weeks on cortisone alone (Swingle, *et al.*, 1952; Howell and Davis, unpublished data) although a low Na and a high K concentration in the plasma frequently occurred even on high doses of cortisone. The finding that a low plasma Na was associated with a negative Na balance (Howell and Davis, unpublished observations) and the present comparative data with DCA suggest that cortisone is a weak Na retaining compound. If ACTH stimulated secretion of adrenocortical salt retaining hormones, failure of salt retention might be an indication that their weak salt retaining action was overbalanced by elevated GFR.

It is evident from the foregoing discussion that the role of the ACTH-adrenocortical system in the regulation of salt metabolism has not been clearly defined. In man, it seems fairly certain that ACTH stimulates secretion of adrenocortical hormones which influence the renal tubular transport of electrolytes (Ingbar and associates, 1951), but in the dog and rat, the situation may differ. In addition to the observations with exogenous ACTH, studies during salt restriction have demonstrated that the response in hypophysectomized dogs differs from that in patients with panhypopituitarism. These patients show impaired ability to conserve

Na; in the report of Stephens (1940), a low plasma Cl (Na not measured) and evidence of acute adrenocortical insufficiency developed within three days. In contrast, the hypophysectomized dogs studied by Earle and coworkers (1951) failed to show a drop in plasma Na during ingestion of a 1 to 2 m.Eq./day Na diet for three weeks. More recently, Rolf and associates (1952) reported that hypophysectomized dogs on a low Na intake lost slightly more Na than normal dogs, but 2 to 5 weeks were required before plasma Na fell. These experiments and studies with exogenous ACTH indicate that anterior pituitary control of adrenocortical salt regulating function differs in dog and man. Additional observations of electrolyte metabolsim in the hypophysectomized dog are needed to determine the exact nature of this difference. Cytochemical studies of the adrenal cortex in the dog under circumstances similar to those reported for the rat (Deane, 1949) may also help to clarify this important aspect of anterior pituitary and adrenocortical physiology.

SUMMARY

The response to ACTH, cortisone, and DCA was studied successively for variable periods in four female mongrel dogs. GFR and RPF increased with all three hormones (except with ACTH in one dog and DCA in another). The immediate effect of these hormones may be a reflection of an action on the renal arterioles. Water exchange was increased by all three preparations. The diabetes insipidus-like state produced by DCA was dependent upon a high Na intake, whereas increased water turnover of lesser magnitude occurred with ACTH and cortisone on both low and high Na diets. A negative K balance resulted with ACTH, cortisone, and DCA but marked K depletion and alkalosis accompanied DCA and a high salt intake only. Fecal K excretion was increased greatly by DCA and slightly by ACTH (3 of 4 dogs) but cortisone was without effect. The first 1 or 2 days of ACTH and cortisone administration were accompanied by salt loss in 3 of the 4 dogs; since GFR was initially increased in these three but not in the fourth animal, a causal relationship between elevated GFR and the negative balance is suggested. Salt retention resulted during the corresponding period on DCA. Thereafter, balance was maintained during administration of all three hormones in spite of a high GFR. Although it is uncertain whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules, the effect of ACTH and cortisone on salt excretion in the dog differs from that reported for man.

Acknowledgment

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STUDIES ON THE INTERRELATIONSHIPS OF THE ADRENAL CORTEX AND RENAL AMMONIA EXCRETION BY THE RAT[1]

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IT HAS been demonstrated recently that the renal capacity of the adrenalectomized animal to secrete ammonium and hydrogen ions is deficient under normal conditions and under conditions of moderate to severe acid stress (Sartorius *et al.*, 1952). Failure to salvage adequately fixed base from the tubular urine in exchange for hydrogen or ammonium ions, results not only in increased loss of base but in a disturbance of body acid base balance. These facts probably account for the chronic acidosis and the poor tolerance to acid stress commonly exhibited by animals with adrenal insufficiency.

The present study was undertaken (a) to determine the capacity of normal and adrenalectomized animals to excrete ammonia and titratable acid when acutely subjected to mild and severe acid stress, (b) to assess the effects of desoxycorticosterone acetate and adrenal cortical extract on ammonia and titratable acid excretion by the adrenalectomized and normal rat, (c) to study the early excretory rates of these ion species after adrenalectomy in an effort to separate or to further link the renal tubular exchange mechanisms, and (d) to ascertain whether acidosis stimulates the adrenal cortex directly as evidenced by a lowering of the glandular content of cholesterol and of ascorbic acid.

METHODS

All experiments were performed on female Sprague Dawley rats weighing from 200 to 250 grams. Animals were bilaterally adrenalectomized in one stage and were allowed a recovery period of 7 to 10 days or more, except where otherwise specified. The operative procedure for the sham operated rats was the same as for those adrenalectomized except that following exposure and mobilization the glands were replaced to their normal position. Adrenalectomized rats were maintained on a high salt diet without further supporting therapy. During the course of each experiment, animals were kept in wire mesh metabolism cages, so constructed as to prevent contamination of the urine with food or feces. In all acute experiments urine was expressed from the bladder by slight supra-

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pubic pressure and collected under oil in small-necked containers in order to reduce evaporating surface. Adequate urine flows were obtained in acute experiments by the administration of 2 cc. of distilled water per 100 grams of body weight 15 minutes prior to the experiment. Water or ammonium chloride as a 5% solution was administered by stomach tube. Desoxycorticosterone acetate in sesame oil was injected subcutaneously in dosages of a half a milligram per rat, 18 hours prior to and 1 hour prior to the experiment.

Urine pH was determined with a Cambridge Glass Electrode, titratable acid by electrometric titration of the urine to a pH of 7.40, urinary ammonia by Conway's microdiffusion method (1942), adrenal cholesterol according to the method of Schoenheimer and Sperry (1934), and adrenal ascorbic acid by the method of Roe and Kuether (1943).

RESULTS

In order to quantitate more precisely the relative deficiencies in capacity of the adrenalectomized rat to secrete ammonia and titratable acid, acute experiments were performed on normal, 2 weeks adrenalectomized, and 2 months adrenalectomized rats subjected to mild and severe acidosis. Production of a mild dilution acidosis was accomplished by the administration of water alone in dosage of 2 cc. per 100 grams of body weight. A more severe acidosis was effected by the administration of ammonium chloride in dosage of 100 mg. per 100 grams of body weight dissolved in the same volume of fluid. Following an initial control period of one hour duration the acid load was imposed and the urine collected for four successive hourly periods. The numbers of rats used in any experiment are indicated in parentheses on each chart.

As is evident in Figure 1, normal rats excreted approximately 11.6 microequivalents of ammonia per 100 grams of rat per hour during the control period. With administration of water alone and the consequent production of a mild dilution acidosis, ammonia excretion rose gradually to a peak of 16 microequivalents. With the added stimulus of an acidifying agent, the excretory rate rose sharply, reaching an average level of 74.3 microequivalents for the fifth hour. In sharp contrast with these levels are those of the adrenalectomized animals. From initial control levels of but 6.6 microequivalents of ammonia per 100 grams of rat per hour, there occurred an increase to but 8.9 microequivalents under water loading and a rise to only 41.1 microequivalents with acid loading. It is thus apparent that although the adrenalectomized rat is capable of increasing ammonia output under stimulation, there is a marked deficiency in its capacity to do so. That this deficiency is not dependent upon, or altered by the length of time following operation is suggested by the fact that two weeks post-adrenalectomized rats and the same group six weeks later exhibited practically identical excretory rates for both ammonia and titratable acid.

Titratable acid excretory rates for both normal and adrenalectomized rats are shown in the upper right hand portion of Figure 1. From control rates of less than 0.2 microequivalent excreted per 100 grams of rat per hour, normal rats responded with an increase to 4.1 microequivalents when given a water load and to an average level of 24.4 microequivalents in the fifth hour when acid loaded. A marked deficiency in the capacity of the adrenalectomized rat to secrete hydrogen ions is apparent, for the maximum excretion with water loading was 0.64 microequivalent while with the imposition of an acid load an excretory rate of only 8.4 microequivalents was attained. The higher urinary pH of the adrenalectomized rat likewise reflects this defect.

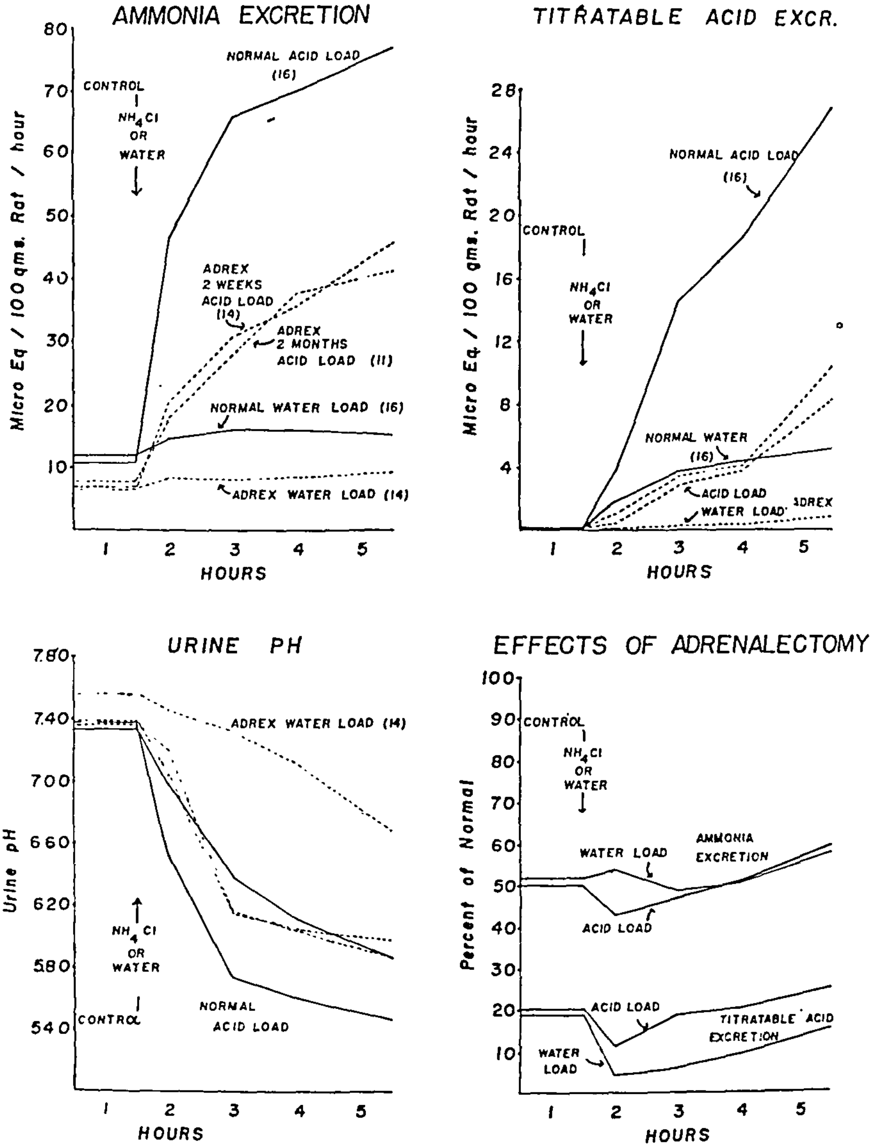

FIG. 1. The effects of adrenalectomy on the capacity of the acidotic rat to secrete ammonia and titratable acid.

In the lower right hand corner of Figure 1, the deficiency in capacity of the adrenalectomized rat to produce ammonia and titratable acid is made more apparent by plotting the output of these substances as a per cent of normal. It is quite evident that the adrenalectomized rat is capable of producing ammonia at a rate of only 50–60% of that of the normal animal, and that this deficiency remains relatively constant for both control and acidotic periods. The capacity of the adrenalectomized rat to form titratable acid is even more limited, amounting to but 15 to 25% of that of the normal animal.

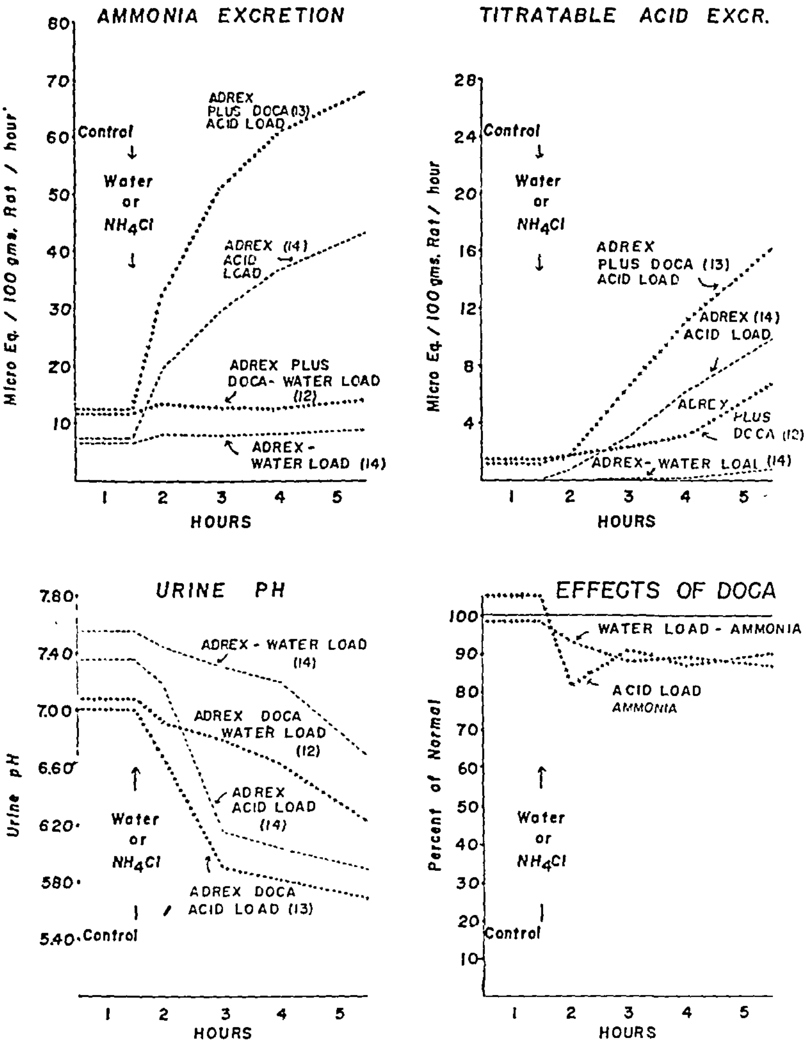

FIG. 2. The effect of desoxycorticosterone acetate on the capacity of the acidotic and adrenalectomized rat to secrete ammonia and titratable acid.

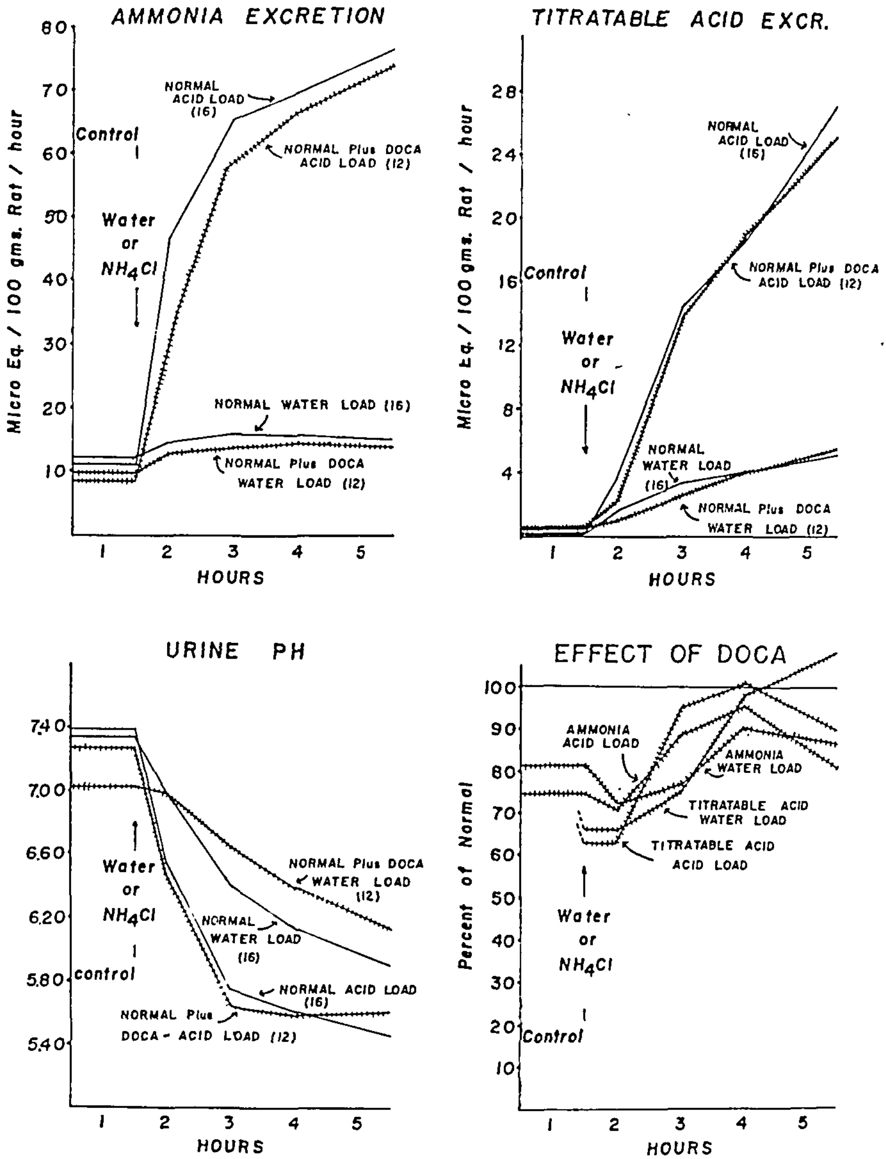

FIG. 3. The effects of desoxycorticosterone acetate on the capacity of the normal but acidotic rat to secrete ammonia and titratable acid.

Since an approximate quantitation of the reduced capacity of the adrenalectomized rat to secrete ammonium and hydrogen ions proved feasible by the administration of water or an acidifying agent in acute experiments, this technique was followed in assessing the activity of desoxycorticosterone acetate on the output of these ions species. Accordingly desoxycorticosterone was administered subcutaneously in dosage of 0.5 mg. per rat 18 hours prior to and again 1 hour prior to each experiment. The effects of the administration of his hormone to adrenalectomized rats are shown in Figure 2. It is apparent that desoxycorticosterone increased appreciably the renal output of ammonia and titratable acid, in the control periods, and in the presence of a mild or severe acidosis. The similarity of the excretory rates of these ions by the adrenalectomized rat receiving desoxycorticosterone with the rates of the normal rat as shown in Figure 1 is striking. The fact that there is almost complete restoration to the normal is seen in the lower portion of Figure 2 where it is apparent that the adrenalectomized rats receiving desoxycorticosterone have increased their capacity to secrete ammonia some 40% or to within 90 to 100% of the normal. Titratable acid is not represented on this chart since desoxycorticosterone increased the control rate of excretion several fold in comparison with the normal. However, the restoration of acid secretory function, though considerable, is not so complete as that of ammonia secretion.

With administration of desoxycorticosterone to the normal rat no increase in ammonia or titratable acid output is apparent. Indeed, almost identical excretory rates were obtained (Fig. 3). In two additional experiments on adrenalectomized and normal rats, adrenal cortical extract in dosage of 1 cc. per rat was administered subcutaneously 18 hours prior to and again 1 hour prior to the start of the experiment. No significant difference was found between the effects of adrenal cortical extract and desoxycorticosterone on ammonia and titratable acid excretion.

Inasmuch as 1) the adrenalectomized animal exhibited a greater capacity to secrete ammonia than hydrogen ions, and 2) desoxycorticosterone acetate or adrenal cortical extract was found capable of restoring these capacities to normal, it seemed profitable to investigate the temporal appearance of these deficiencies after acute bilateral adrenalectomy. Accordingly, experiments were performed in which all animals were either sham operated or adrenalectomized after the collection of two control periods. At the time of the operative procedure the animals were given 2 cc. of water per 100 grams body weight or ammonium chloride in dosage of 1 mg. per gram of body weight in a similar amount of water. In those experiments involving the administration of an acid load, a similar dose of ammonium chloride was administered 42 hours prior to, 24 hours prior to, and 18 hours prior to the start of the experiment. Following the operative procedure, urine was collected at 2 hour intervals for 12 successive collection periods.

It is apparent that ammonia and titratable acid excretory rates for both the sham operated and adrenalectomized animals rose sharply following the administration of an acid load (Fig. 4). The adrenalectomized rats, however, attained peak excretory rates at an earlier time and at lower values than did the intact rats. As was expected, the deficiency in capacity of the adrenalectomized rat to excrete titratable acid was of greater magnitude than the deficiency in capacity to excrete ammonia. With administration of a water load, similar deficienceis were noted.

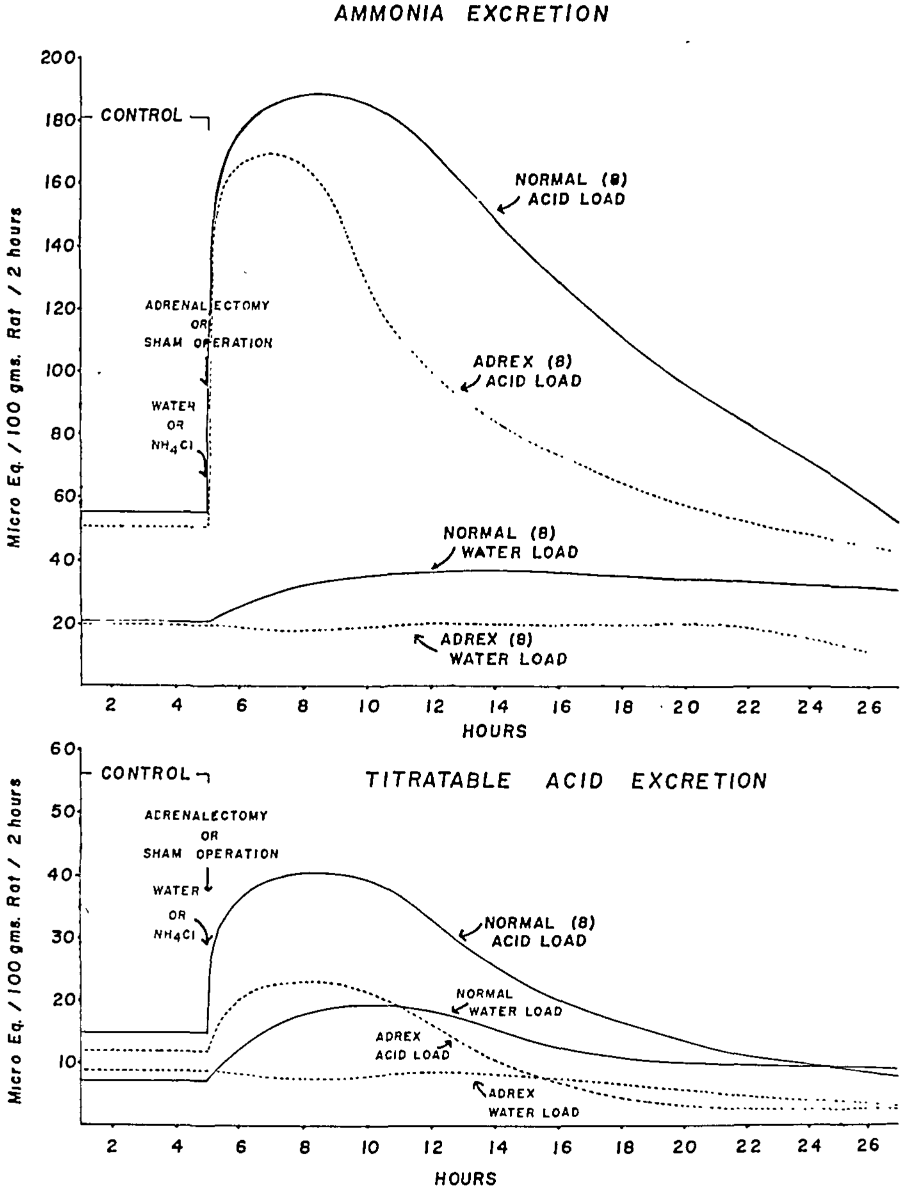

FIG. 4. The early time courses in the capacities of the sham-operated normal and acutely adrenalectomized rat to secrete ammonia and titratable acid.

It remained to be demonstrated that ammonium chloride actually stimulates the adrenal cortex. Evidence for stimulation of the adrenal cortex was obtained by administering ammonium chloride and then periodically assaying the adrenal cortices for their content of cholesterol and ascorbic acid. Urine collections were obtained at similar time intervals in order to compare the time of peak ammonia and titratable acid outputs with the time of minimum glandular cholesterol content.

As may be seen in Figure 5, the average cholesterol content fell from a control level of 0.778 mg. per 100 grams of body weight to a low of 0.514 mg. four hours after the administration of 1 mg. of ammonium chloride per gram of body weight. Peak ammonia and titratable acid excretion occurred six hours after the administration of the acid load. Ascorbic acid content of the adrenal gland fell from control values of 5.5 mg. per 100 grams body weight to a low of 4.54 mg. two hours after the acid loading and then gradually rose to somewhat elevated values.

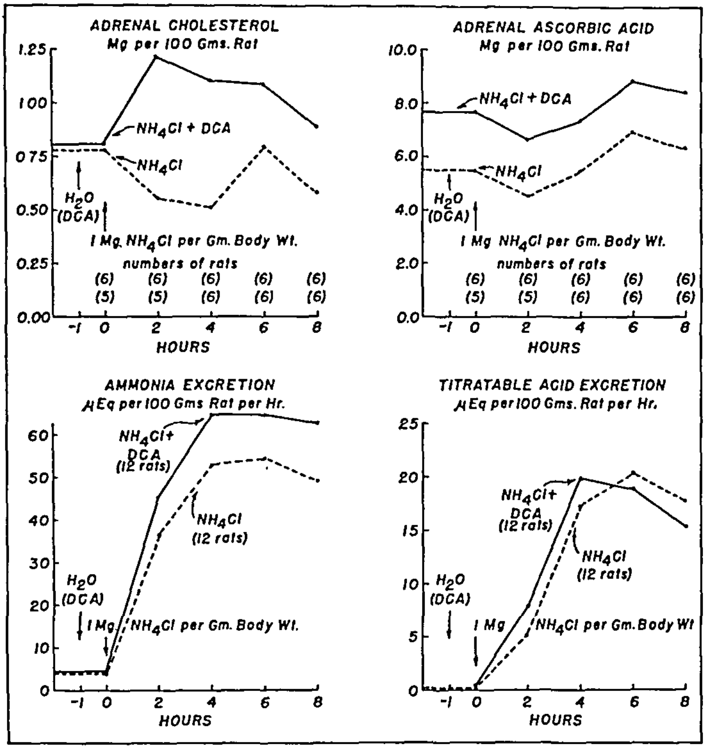

FIG. 5. The effects of ammonium chloride and ammonium chloride plus desoxycorticosterone acetate on the adrenal cholesterol and ascorbic acid content of the rat.

DISCUSSION

It is well established that the untreated adrenalectomized animal loses excessive quantities of sodium and chloride in the urine and in consequence suffers reduction in the plasma concentrations of these ions and in the volume of extracellular fluid as well (Loeb, 1932; Harrop *et al.*, 1933, 1936). However, an additional quantity of base is lost because of a relative deficiency in renal capacity to secrete hydrogen ions and ammonia (Jiminez-Diaz, 1936; Sartorius *et al.*, 1952). This deficiency accounts for the mild to moderate acidosis (reduction in bicarbonate bound base) of the ad-

COMPARISON OF POTENCY OF DIFFERENT ACTH PREPARATIONS BY INTRAVENOUS AND SUBCUTANEOUS ROUTES OF INJECTION

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MANY papers that indicate the existence of multiple ACTH factors have been published. Astwood (1952) and Reinhardt, Geschwind and Li (1951) have reviewed these papers recently. Most of the eivdence for two or more factors is based on quantitative differences in potency of preparations when assayed on the one hand by the ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) after intravenous injection (one hour test) and, on the other hand, by increase in adrenal weight or histological changes after subcutaneous injection (3–4 days).

Because of the differences in experimental method described in these reports, e.g., in route of injection, duration of treatment, and end point observed, the following experiments, in which only the route of injection was varied, were carried out. Hypophysectomized rats were injected subcutaneously or intravenously with two different types of preparations of ACTH. The left adrenal gland was removed one hour and the right adrenal two hours later for determination of the ascorbic acid content. It was found first, that a 100 to 1000 times larger dosage may be required subcutaneously than intravenously to cause a significant ascorbic acid depletion and second, that there was a large difference in the potency estimates with two different types of ACTH preparations depending upon the route of injection.

METHODS

Groups of male rats from Hormone Laboratories, Chicago, approximately 40 hours after hypophysectomy were injected either intravenously (nembutal anesthesia) or subcutaneously (no anesthesia) with 0.5 ml of aqueous solution of ACTH at 3 or more appropriate dosage levels, which cause a 20–50% ascorbic acid depletion. Under ether anesthesia, at one hour after injection, the left adrenal was removed, trimmed, weighed and placed in metaphosphoric acid solution. Two hours after injection the rats were killed with chloroform and the right adrenal removed and treated as was the left adrenal. Ascorbic acid content was determined by the method of Mindlin and Butler (1938). There were at least 3 rats at each dosage level.

ACTH PREPARATIONS

Frozen hog pituitary glands were ground and extracted twice at pH 2.8 using sulfuric acid and a solvent medium of 20% ethanol. The combined

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extracts were concentrated in vacuo to a volume of about 100 ml./lb. and an equal volume of acetone added. The precipitates that formed were removed and found to have little potency. The clear 50% acetone solution was run through an IR-120 column in the acid cycle. To the acid effluent from the column with a pH of about 1.6, two volumes of anhydrous acetone were added slowly, with stirring, making the acetone concentration about 84% by volume. The gummy protein precipitate which formed, was redissolved in a minimum volume of water and poured into 20 volumes of acetone. The *flocculent white precipitate of ACTH was removed by* decantation and centrifugation and thoroughly dried with acetone. Such preparations in general are soluble at all pH's in H_2O and have a potency of 2–6 I.U./mg. They are indicated as A preparations in Table 1 and Figures 1 and 2. P 327A had a higher potency because it had been refractionated in acid acetone.

TABLE 1. SHOWING THE RELATIVE POTENCY OF ACID, A, AND BASIC, B, ACTH PREPARATIONS WHEN ASSAYED BY TWO ROUTES OF INJECTION

(1) Preparation	(2) Dosage to cause 20% A. A. depletion		(3) Dosage ratio Subcut./I.V. from Col. (2)	(4) Ratio of A/B from Col. (3)
	I.V. µg.	Subcut. µg.		
E 3A	0.33	100	300	3
E 13B	0.33	33	100	
M 2160A	0.5	100	200	11.1
M 2161B	1.5	27	18	
P 327A	0.04	50	1250	10.4
D 13B	0.25	30	120	
C 12A	0.1	125	1250	6
C 15B	0.2	50	250	

The gland residue, after the second extraction above, was suspended in ethanol in an amount that resulted in an ethanol concentration of 65–70% by volume and NaOH added until the pH (glass electrode) was 9.5–10. The third and fourth extracts were made under these conditions, which were those used by Bates and Riddle (1935) to extract prolactin from bovine pituitaries. The extracts were neutralized to pH 6 and 2 volumes of ethanol added to precipitate the ACTH and other proteins. The precipitate was then extracted twice with 50% acetone at pH 9 and the extract adjusted to pH 3 where another precipitate was removed and re-extracted. The pH 3, 50% acetone soluble fraction was then deashed by use of an IR-120 resin column as for the first and second extracts but the effluent was made up to 90–95% acetone at once. The white precipitate was dried and contained the ACTH from the third and fourth or alkaline extracts.

Such preparations are insoluble in water between pH 3.5 and 6.5 and usually have a potency of 1 to 2 I.U./mg. They are indicated as B preparations in Table 1 and Figures 1 and 2.

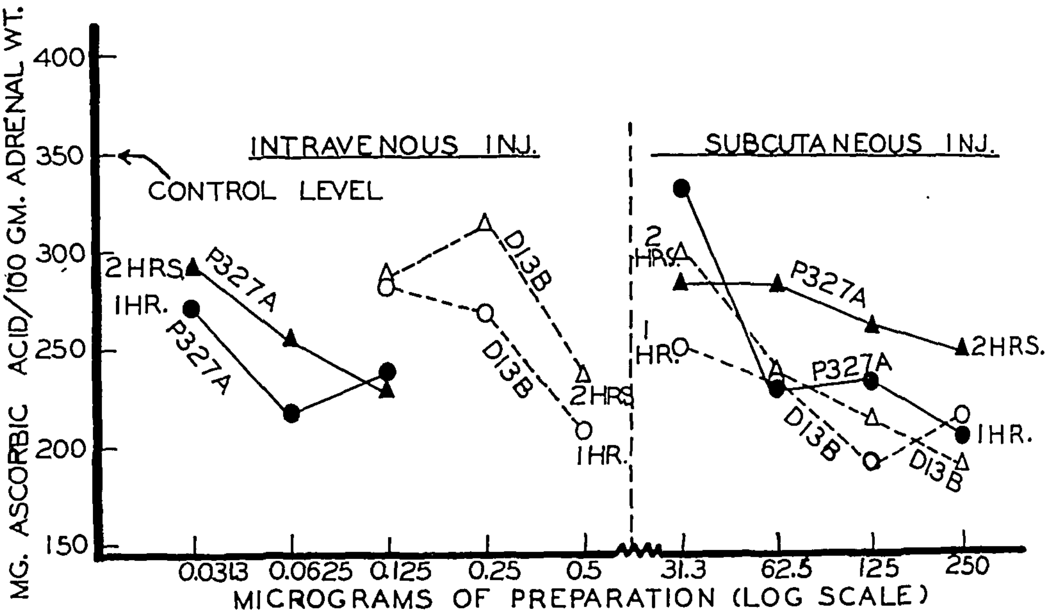

FIG. 1. Showing quantitative differences between two ACTH preparations injected by different routes.

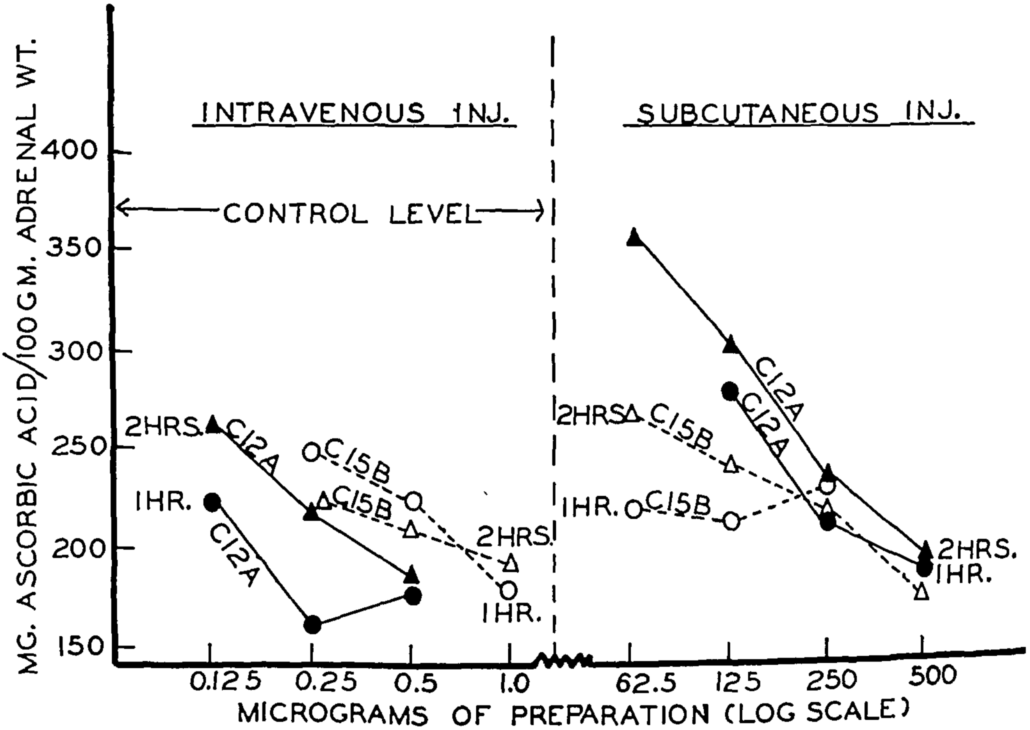

FIG. 2. Showing quantitative differences between two ACTH preparations injected by different routes.

RESULTS

Four comparisons were made between A extracts which were acid extracts and B extracts which were basic or alkaline extracts. A comparison of the effect of two pairs of these preparations upon the ascorbic acid levels in the adrenal when injected intravenously and subcutaneously are shown graphically in Figures 1–2. In both cases it can readily be seen that the A fractions are more potent than the B fractions by intravenous injection. In contrast, when given subcutaneously, the B preparations cause an equal or greater depletion of ascorbic acid than the A preparations.

Another highly significant fact shown in these figures is that the subcutaneous dosage required to cause 20–50% depletion of ascorbic acid is 100 or more times greater than the intravenous dosage. It is apparent from the figures that the slopes of the dosage response lines with different preparations are not always parallel especially when the subcutaneous route of injection is used.

In general, the ascorbic acid levels of the adrenals two hours after injection are slightly higher than after one hour indicating that the adrenal ascorbic acid level is beginning to return towards the control level. The ascorbic acid levels at the two time intervals are confirmatory of each other so that the comparisons may be considered as being carried out in duplicate. The two time intervals were used because we had previously found that the fall in ascorbic acid level after ACTH was injected intravenously was maximum in 10 to 15 minutes whereas it was not maximum until about one hour after ACTH was injected subcutaneously. It was thought desirable to consider time differences in the experiment due to the different rates of action of the ACTH by the different routes of injection.

By averaging the one and 2 hour data and by graphical interpolation and extrapolation, an estimate was made of the dose to cause a 20% ascorbic acid depletion by the different routes of injection. This is shown in column 2 of Table 1. These estimates, of course, are subject to considerable error due to the small number of animals used, the variability of the amount of depletion in different rats and the differences in slope, especially the tendency for horizontal slope with the subcutaneous route. In our laboratories 0.5 to 1 milliunit of the International Standard, when given intravenously, will cause a 20% depletion of the ascorbic acid level. Thus, preparation P 327A has a potency of 10 to 20 I.U./mg., and D 13B of 2 to 4 I.U./mg. In column 3 it is shown in the case of P 327A that 1250 times more ACTH is required subcutaneously than intravenously to cause a 20% ascorbic acid depletion whereas with D 13B only 120 times as much ACTH is required.

Column 4 shows how much the relative potency of the A and B fractions differ with the different routes of injection. Thus E 3A would be only $\frac{1}{3}$ as potent as E 13B if the potency were determined by the subcutaneous route whereas they are of equal potency by the intravenous route. Likewise P 327A, which is approximately 6 times more potent than D 13B by intravenous assay is only 60% as potent as D 13B by subcutaneous assay making an over-all difference of potency of 10.4 times depending upon whether the intravenous or subcutaneous estimates of potency were used.

An additional experiment was run comparing intraperitoneal and subcutaneous routes of injection. The dosage ratios by the two routes expressed as in Table I, column 3, (Subcut./I.P.) were 1.1 for M 2160A and 1.33 for M 2161B, which values are not significantly different from 1.0.

A preliminary experiment with subcutaneous injection twice daily for four days of P 327A and D 13B into hypophysectomized rats indicated that they were about equal in adrenal weight increasing potency. A 50% increase in adrenal weight was obtained with 6.7 mg. and 3.3 mg. respectively. As would be expected, the adrenal weight increasing potency was more nearly correlated with the ascorbic acid depletion tests when injections were made subcutaneously than when made intravenously.

Unpublished results, using adrenal ascorbic acid depletion in normal rats as the end point, upon duration of action of single subcutaneous injections of 16 I.U. of ACTH formulated in sesame oil in a manner to slow absorption (see also Astwood, 1952), show that ACTH preparations like A and B can be made to increase adrenal weight by over 50% in 24 hours and 100% in 48 hours while maintaining ascorbic acid levels more than 50% below control levels. This remarkable increase in adrenal weight is due to the slow release of ACTH over a period of days, as shown by the ascorbic acid levels, and not to any special qualitative property of the ACTH in the preparations. Numerous clinical reports of the high potency of ACTH given by slow intravenous infusion have been made (Mandel, Singer, Gudmundson, Meister and Modern, 1951; Renold, Forsham, Maisterrana and Thorn, 1951).

DISCUSSION

Astwood and coworkers (1952) have made preparations of ACTH by adsorption on oxycellulose that are 25 to 100 times more potent than the ones used here. Hence, the preparations used here and those used by most other investigators contain 95% or more of non-ACTH protein or protein-like material. The different ascorbic acid depletion potencies of the A and B preparations, shown above, and different solubilities probably are not due to the ACTH itself but are due to differences in the 95% contaminants and their effect upon the rate of absorption from the site of injection.

Different estimates of potency by the different routes of administration are important clinically because most ACTH is used by routes of injection other than the intravenous route which is used for standardization. Assay of ACTH preparations by the universally used intravenous Sayers or Munson methods probably does not properly indicate the clinical efficacy of the preparation when given intramuscularly. Conversely a subcutaneous assay method would not properly indicate the intravenous efficacy of a preparation. In all 4 cases in Table 1 the iso-electrically precipitable fraction, B, is much more potent subcutaneously than the intravenous assay would indicate it to be. Clinical proof that a similar difference between these preparations occurs in man is not available.

Most differences in physiological efficacy of ACTH are as readily explained on the basis of different rates of absorption, which depends upon the route of injection and the nature and formulation of the preparation, as on the basis of a qualitative difference in the ACTH present, i.e., the multiple factor theory.

SUMMARY

The potencies of two different types of ACTH preparations were compared by intravenous and subcutaneous injection using adrenal ascorbic acid depletion in the hypophysectomized rat as the criteria of potency. Estimates of the relative potency of the two types differed with the route of injection by 3 to 11 times. The subcutaneous route of injection required as much as 100 to 1000 times larger dosage than did the intravenous route to cause equivalent ascorbic acid depletion. The data given are interpreted as not supporting the multiple ACTH factor therory.

Acknowledgements

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RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE, AND TESTOSTERONE PROPIONATE COMBINED WITH GROWTH HORMONE

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IN THE human, normal growth appears to occur in two phases: prepuberal growth, hypothesized as being entirely referable to pituitary growth hormone, and puberal growth referable to steroid growth promoting factors from the gonads and the adrenal cortex (Kinsell *et al.*, 1948). Nitrogen retention and protein anabolic effects of testosterone and its derivatives, methyltestosterone and particularly testosterone propionate have received considerable attention, and, although much factual information has been obtained (Kochakian, 1946, 1950), elucidation of the mechanism of these effects is still awaited.

Technical procedures and mathematical treatment developed by Sprinson and Rittenberg (1949) and Hoberman (1949–50) for assessing protein and amino acid metabolism in terms of rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool have been utilized in characterizing and quantitating the over-all mechanisms involved in nitrogen storage induced in the adult female dog with anterior pituitary growth hormone (Bartlett and Gaebler, 1952). The effect of growth hormone in depressing the rate of amino acid catabolism and increasing the size of the nitrogen pool was particularly striking and as previously suggested (Bartlett and Glynn, 1950) perhaps indicative of the mechanism by which growth hormone regulates the intracellular synthesis of protein.

Extension of these studies to include nitrogen storage induced in the adult female dog with testosterone propionate seemed particularly desirable not only from the standpoint of comparing the pattern of the over-all mechanisms involved in the metabolism of proteins and amino acids with the pattern observed during nitrogen storage induced by growth hormone but also because of widespread clinical interest in the use of androgens to promote nitrogen retention. It was felt that information of this sort might also be of value in extending our knowledge and understanding of the role of androgens in the regulation of puberal growth. In

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view of the summation effect of testosterone propionate and growth hormone on body weight and protein anabolism of castrated mice (Kochakian and Stettner, 1948), it also seemed of interest to include experiments in which nitrogen storage was induced by the simultaneous administration of growth hormone and testosterone propionate at dose levels corresponding to those given in single experiments.

EXPERIMENTAL

Animals—Dogs 44, 52, and 55 utilized in the preceding study with growth hormone (Bartlett and Gaebler, 1952) and maintained in the manner previously described (Gaebler, Bartlett and Sweeney, 1951) were used in the experiments reported herein.

Procedure—Since data were available on the rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool for each of these animals during nitrogen balance experiments (Bartlett and Gaebler, 1952), it was only necessary to conduct experiments in which nitrogen storage was induced with testosterone propionate or the combination of testosterone propionate with growth hormone. The experimental procedure followed and the mathematical treatment of the data were essentially the same as previously described. Constancy of both body weight and total urinary nitrogen excretion were taken as indicative of nitrogen equilibrium and nitrogen storage was induced in such animals either by the intramuscular injection of testosterone propionate at dose levels of 50 mg./day for 5 days, or by a combination of this level of androgen with the subcutaneous administration of growth hormone in doses of 5 mg./day for 5 days. N^{15}-labeled glycine was administered on the 4th day of induced nitrogen storage experiments and urinary excretion of the N^{15} label determined at 24 and 48 hours after ingestion of the amino acid.

RESULTS

In Table 1 are summarized the results of experiments in which nitrogen storage was induced with testosterone propionate. The rate of protein synthesis, S_k, and the size of the nitrogen pool, P_k, were calculated according to the mathematical treatment of Sprinson and Rittenberg (1949), and, k_1 and k_2, rates of amino acid catabolism and incorporation of amino acids into protein, by the mathematical treatment of Hoberman (1949–50). S_k values previously reported (Bartlett and Gaebler, 1952) for Dogs 44, 52, and 55 during nitrogen balance experiments were respectively in contrast to those given in Table 1—0.49, 0.56, and 0.82 gm. N/kg. body wt./24 hrs., and P_k values were respectively 0.82, 0.91, and 0.95 gm. N/kg. body wt. Values of k_1 abstracted from the same reference were respectively 3.85, 3.96, and 2.74%/hr. and values for k_2 3.85, 4.19, and 3.60%/hr. In general, the rate of protein synthesis was higher and the rate of amino acid catabolism lower during nitrogen storage induced with testosterone propionate than during the nitrogen balance experiment. There appeared to be no significant change in the size of the nitrogen pool, P_k.

Results of experiments in which nitrogen storage was induced in Dogs 44 and 55 by the combination androgen and growth hormone treatment are presented in Table 2. Comparison of this data with the nitrogen balance

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF THE NITROGEN POOL

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N: Catabolized and excreted, k_1 (%/hr.)	Fraction of amino acid N: Incorporated into protein k_2 (%/hr.)
44	0.31	3.89	0.65	0.82	2.18	4.70
52	0.14	2.66	0.79	0.81	3.19	5.51
55	0.43	3.40	0.66	1.05	2.38	3.52

[1] Nitrogen storage induced in Dogs 44, 52 and 55 with Schering's Oreton brand of testosterone propionate.

[2] Weight gain was computed by determining the difference between the maximum weight attained during the 5 day period of induced nitrogen storage and the weight of the animal preceding induction of nitrogen storage.

[3] Nitrogen storage was calculated from total urinary nitrogen excretion alone. The average of 5 or more days preceding induction of nitrogen storage was taken as the control level and the difference between this value and the urinary nitrogen excretion for each day of the 5 day period determined. The values given in the table are summation values for the 5 day period of storage.

data from the same animals results in a number of interesting observations. In spite of the induction of marked nitrogen storage and gain in body weight as a result of the combined treatment with androgen and growth hormone, the rate of protein synthesis calculated in terms of either S_k or k_2 was not appreciably affected. Rates of amino acid catabolism were decreased in both animals and the size of the nitrogen pool was reduced.

TABLE 2. RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM, AND SIZE OF THE NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE PLUS GROWTH HORMONE

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N: Catabolized and excreted, k_1 (%/hr.)	Fraction of amino acid N: Incorporated into protein, k_2 (%/hr.)
44	0.71	10.88	0.47	0.58	1.86	3.94
55	0.79	11.18	0.74	0.87	2.28	4.30

[1] Nitrogen storage was induced in Dog 44 with testosterone propionate (Oreton) combined with growth hormone preparation Rx099916, supplied through the courtesy of Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis & Company, and, in Dog 55 with testosterone propionate (Oreton) combined with growth hormone preparation 22KR1 supplied by Armour and Company.

[2,3] *Please refer to footnote of Table 1.*

DISCUSSION

The observation of a decrease in the rate of amino acid catabolism, k_1, in the normal adult female dog during nitrogen storage induced with testosterone propionate is in agreement with the observation that decreased urinary urea excretion in this animal is not due to elevated blood nonprotein nitrogen (Gaebler and Tarnowski, 1943). In terms of rates of protein synthesis and amino acid catabolism, nitrogen storage induced in the adult female dog with testosterone propionate appears to be qualitatively similar to nitrogen storage induced with growth hormone (Bartlett and Gaebler, 1952), *i.e.*, characterized by a higher rate of protein synthesis and a lower rate of amino acid catabolism than observed in the same animal during nitrogen balance. Testosterone propionate in contrast to growth hormone appears to have no significant effect on the size of the nitrogen pool.

In considering changes in the rates of the principal over-all reactions required to either increase or decrease the size of the metabolic pool of nitrogen, relative to nitrogen balance values, a decreased rate of protein synthesis or amino acid catabolism or an increased rate of protein degradation might singly or in one of several combinations result in a net increase in the size of the nitrogen pool, while an increased rate of protein synthesis or amino acid catabolism or a decreased rate of protein degradation might similarly result in a net decrease in the size of the nitrogen pool. In view of the fact that the pattern of protein metabolism in terms of rates of protein synthesis and amino acid catabolism is qualitatively similar in nitrogen storage induced by either growth hormone or testosterone propionate, the contrast in the net effect on the size of the nitrogen pool seems of special interest. The complexity of the situation and the difficulty involved in its interpretation is emphasized with the realization that the net effect on the size of the nitrogen pool could, in addition to those factors previously noted, also be determined by either the extent to which the rate of protein synthesis was increased and the rate of amino acid catabolism decreased or by changes in the rate of protein degradation or by a combination of these contributing factors. The importance and necessity of having data on the relative rates of protein degradation during both nitrogen balance and nitrogen storage experiments seems quite apparent, and until such data are available, progress in the elucidation of the mechanisms involved in nitrogen storage and growth and particularly in respect to the accumulation of protein will be limited.

The observation that growth hormone (Bartlett and Gaebler, 1952) and testosterone propionate administered alone increased the rate of protein synthesis, but when administered in combination had no significant effect on this value, and the observation that the rate of amino acid catabolism was decreased are of special interest. The fact that the size of the nitrogen pool actually decreased under these conditions suggests that the combination androgen and growth hormone treatment resulted in a significant reduction in the rate of protein degradation.

In connection with the hypothesized endocrine regulation of prepuberal and puberal growth (Kinsell *et al.*, 1948), it should be noted that growth during the puberal period, which is particularly characterized by the accumulation of body protein (*e.g.*, in the development of the musculature), might also be referable to a combination of growth hormone and steroid growth promoting factors such as the androgens and estrogens. If such is the case, and, the results of our experiments in which nitrogen storage was induced in the adult female dog with growth hormone and in combination with testosterone propionate can be interpreted respectively in terms of simulated prepuberal and puberal growth then the latter in contrast to prepuberal growth may be largely due to a decrease in the rate of protein degradation. The relatively high protein requirements and output of nitrogen during the phase of prepuberal growth as compared with that during puberal growth might be taken as evidence supporting the view that prepuberal growth is characterized by a high level of protein metabolism, *i..e.*, a high rate of protein synthesis and a high rate of protein degradation, whereas, puberal growth is characterized by a relatively lower level of protein metabolism, *i.e.*, relatively lower rates of protein synthesis and protein degradation.

SUMMARY

Nitrogen storage induced in the adult female dog with testosterone propionate is characterized by an elevation in the rate of protein synthesis and a decrease in the rate of amino acid catabolism relative to values observed in the same animal during nitrogen balance experiments.

Testosterone propionate had no significant effect on the size of the metabolic pool of nitrogen in the adult female dog.

A combination of growth hormone and testosterone propionate produced marked nitrogen storage and gain in body weight, a decrease in the rate of amino acid catabolism and the size of the nitrogen pool in the adult female dog. The rate of protein synthesis was unaffected in the same animal.

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THE INTERCONVERSION OF ESTRONE AND ESTRADIOL-17β BY RAT LIVER SLICES[1,2,3]

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SINCE Zondek's original demonstration (1934) that rat liver mince "inactivates" estrogens, many workers have engaged in the study of the *in vitro* metabolism of these steroids employing loss of biological activity as a means of following hormone degradation (Heller, 1940; Engel, P. and Rosenberg, 1945; Twombly and Taylor, 1942; Levy, 1947 and DeMeio *et al.*, 1948). Pearlman and DeMeio (1949) accomplished the only isolation of estrone after the incubation of estradiol-17β[5] with rat liver slices. A technique for the isolation and determination of estrogens in minute quantities would be of great assistance in studying this reaction in detail. Counter-current distribution and fluorometric analysis have provided a means for the separation, quantitative estimation and identification of natural estrogens in urinary extracts (Engel *et al.*, 1950). This report describes the application of these techniques to the analysis of estrogens after incubation with rat liver slices.

METHODS

Tissue Preparation: Female Wistar strain rats, 6–10 months old were decapitated and bled. Their livers were quickly removed, chilled, sliced to 0.5 mm. thickness with a Stadie-Riggs microtome, drained and weighed. Cirrhosis and hepatomas were induced in similar rats by the administration of a high fat diet containing p-dimethylaminoazobenzene (Kline *et al.*, 1946) and prepared for incubation as described above. When hepatomas were used for incubation, they were dissected free from surrounding tissue. Microscopic sections were prepared and examined to insure purity of the preparation.

Hormone Preparation: Estradiol was analyzed by the counter-current, fluorometric

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[2] A preliminary report of this work was presented at the Second International Congress of Biochemistry, Paris, France, July 21–26, 1952.

[3] This is publication #777 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

[5] Hereafter referred to as estradiol. Nomenclature employed is that of Fieser and Fieser (1949).

and estradiol were incubated with boiled liver and an average recovery of 76% was obtained (Fig. 2). Owing to changes in the physical state of the tissue proteins, the extraction of estrogens from boiled liver is rendered more difficult.

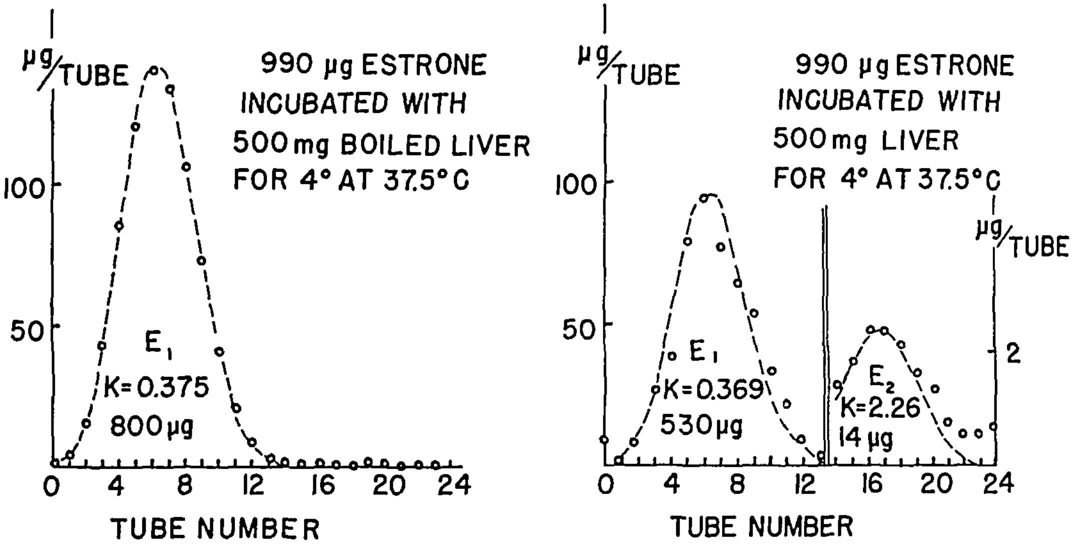

FIG. 2. Incubation of estrone with rat liver slices. Typical counter-current distribution curves. Intact liver slices converted estrone to estradiol. Boiling the tissue prevented conversion. E_1—Estrone; E_2—Estradiol; K—Partition Coefficient; ∘—Experimentally determined points; - - - —Theoretical.

TABLE 1. EFFECT OF VARYING THE HORMONE SOLVENT ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Solvent	Initial estradiol	Total recovery	Estradiol recovered		Estrone yield		
	µg	%	µg	K*	µg	%	K*
Aqueous	970	46%	430	2.1	18.5	1.9	0.34
	970	63	605	2.2	9.2	0.9†	0.31
	970	29	276	2.0	7.5	0.8	0.36
	490	64	294	2.1	18.2	3.7	0.39
	900	43	365	2.1	22.0	2.5	0.38
Ethanol	1020	64	555	2.1	93.0	9.1	0.33
	1020	26	222	2.1	39.8	3.9	0.36
	1020	22	188	2.3	36.1	3.5†	0.36
	1020	48	440	2.0	47.0	4.6	0.36
	1020	68	633	2.2	61.2	6.0‡	0.35
Aqueous with added Ethanol	980	37	336	2.0	21.4	2.2	0.39
	660	46	260	2.1	38.3	5.8	0.41
	750	35	233	2.1	31.0	4.2	0.38

* K—Partition coefficient in 50% MeOH—50% H_2O/CCl_4
†—2 hour incubation.
‡—Intracellular buffer.

Incubation of Estradiol with Normal Liver Slices (*Table 1*): When estradiol in ethanol solution was incubated with normal rat liver slices using a hormone concentration of 0.02 mg./mg. tissue/ml. buffer, there was an average yield of 5.4% estrone and a recovery of 40% of unchanged estradiol. This is in close agreement with the data of Pearlman and DeMeio (1949) who isolated 6% estrone and recovered 40% estradiol after incubation of the hemisuccinate of estradiol with rat liver slices. The 55% of starting material unaccounted for represents conversion of estrone and/or estradiol to unknown metabolites.

TABLE 2. EFFECT OF VARYING THE HORMONE CONCENTRATION ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Hormone concentration*	Initial estradiol µg	Total recovery %	Estradiol recovered		Estrone yield		
			µg	K	µg	%	K
0.1	5100	72	3630	2.2	51	1.0	0.35
	1020	45	450	2.1	14	1.4	0.37
0.02 (Aver. of 3 runs Table I)	1020	46	408		54	5.9	
0.006	1020	33	191	2.1	147	14	0.37
	1020	0.3	1.8	2.1	1.2	0.1	0.37

*—Hormone concentration = mg. hormone/mg. liver/ml. buffer.

An experiment employing an intracellular buffer is listed in Table 1 but the results are not strikingly different from those obtained with the conventional Krebs' solution. Results similar to these have been reported by DeMeio *et al.* (1948).

In the experiments cited above, alcohol was used as solvent to disperse the estradiol in the buffer solution. The amount of ethanol used was similar to that employed by DeMeio (1948). To test the possible effect of the ethanol on the metabolic reaction, the sodium salt of estradiol was prepared and added to the buffer solution. When the pH was adjusted to 7.4 the hormone was precipitated as a fine dispersion. The results of using this aqueous dispersion are shown in Table 1. When an 0.02 hormone concentration was used, the average yield of estrone was 2.7% and the recovery of estradiol was 49%. If the incubations were carried out with aqueous dispersions of the steroids (prepared from the sodium salts) to which ethanol was added, the yield of estrone was increased to 4% and the recovery of estradiol was 39%. The amount of ethanol was equal to the quantity used in the experiments in which this solvent alone was employed.

When the hormone concentration was increased to 0.1, the yield of estrone fell to 1%. Changing the hormone concentration to 0.006 increased both the destruction of estradiol and the yield of estrone (Table 2). In one experiment of this series, less than 1% of the steroid was recovered (cf. Pearlman and DeMeio, 1949). These data on the effect of changing hormone concentration are based on a small number of experiments in which there is a great deal of variation; nevertheless, a trend is apparent.

TABLE 3. AEROBIC CONVERSION OF ESTRADIOL* TO ESTRONE BY DISEASED RAT LIVER

Pathology	Hormone concentration	Total recovery %	Estradiol recovered		Estrone yield		
			μg	K	μg	%	K
Cirrhosis	0.02	74	735	2.0	13.1	1.3	0.33
	0.02	64	605	2.0	40.5	4.0	0.35
Hepatoma	0.02	80	805	2.1	5.9	0.6	0.33
	0.02	41	407	2.0	10.3	1.0	0.31
	0.02	70	710	2.3	6.8	0.7	0.35
Cirrhosis	0.006	60	470	1.8	138	14	0.33
	0.006	16	106	2.1	60	5.9	0.36
	0.006	1.5	0.8	2.0	0.7	0.07	0.42
Hepatoma	0.006	7.0	4.0	2.2	3.0	0.3	0.34

*—Initial estradiol = 1 mg.

TABLE 4. ANAEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY RAT LIVER SLICES

Solvent	Initial estradiol μg	Total recovery %	Estradiol recovered		Estrone yield		
			μg	K	μg	%	K
Aqueous	490	96	440	2.1	31.9	6.5	0.36
Ethanol	540	72	376	2.1	13.2	2.5	0.38

Incubation of Estradiol with Cirrhotic Livers and Hepatoma Nodules (Table 3): When estradiol was incubated with slices of cirrhotic liver or with hepatoma nodules using a hormone concentration of 0.02 mg. hormone/mg. liver/ml. buffer the yield of estrone was much lower and the recovery of estradiol was somewhat higher than with normal tissues. When the hormone concentration was decreased to 0.006, the difference between normal, cirrhotic and malignant tissues become less marked. The variations observed in the cirrhotic livers may be attributed to differences in the severity of the disease.

Anaerobic Experiments (Table 4): When estradiol in aqueous solution was incubated in evacuated Thunberg flasks, there was some destruction of the estradiol and conversion to estrone.

TABLE 5. CONVERSION OF ESTRONE TO ESTRADIOL BY NORMAL RAT LIVER SLICES

Solvent	Gas phase	Initial estrone µg	Total recovery %	Estrone recovered µg	Estrone recovered K	Estradiol yield µg	Estradiol yield %	Estradiol yield K
Ethanol	air	990	60	585	0.37	14.1	1.4	2.1
	air	990	55	530	0.37	13.8	1.4	2.3
Aqueous	air	900	45	620	0.38	2.0	0.2	2.1
Aqueous with added Ethanol	air	950	52	480	0.38	10.0	1.1	2.1
Ethanol	N_2	495	82	380	0.35	24.5	5.0	2.2

Incubation of Estrone: When estrone was incubated with rat liver slices in air or nitrogen, conversion to estradiol occurred by the yield depended on whether an aqueous or ethanol solution was used (Table 5). An experiment employing an aqueous solution of estrone with added ethanol gave results similar to those found with alcohol alone. These findings are similar to those obtained with estradiol under these varying conditions (Table 1).

DISCUSSION

Methods: In an effort to obtain quantitative data and study subtle changes, counter-current distribution and fluorometry have been employed in the present work. It has been possible by these methods to characterize as little as 1 microgram of estrogen as either estradiol or estrone and to reproduce partition coefficient within standard diviations of ±0.02 for estrone and ±0.1 for estradiol for hormones extracted from incubation mixtures (Table 6). From the distribution data, it is possible to calculate the amounts and purities of the components present and in addition characterize them by their partition coefficients (Engel, Slaunwhite, Carter and Nathanson, 1950).

Interconversion of Estradiol and Estrone: The data presented in Tables 1 and 5 demonstrate the interconversion of estradiol and estrone. Under the

TABLE 6. REPRODUCIBILITY OF PARTITION COEFFICIENTS BY COUNTER-CURRENT DISTRIBUTION—24 TRANSFERS IN 50% $MeOH/CCl_4$

Compound recovered	Number of distributions	Partition coefficient	Standard deviation	Extracted from	Reference
Estrone	85	0.36	±0.02	Incubated tissue	Present study
	29	0.34	±0.02	Urine	(Slaunwhite)
	—	0.33	—	Pure compound	(Engel, 1950)
Estradiol	80	2.1	±0.1	Incubated tissue	Present study
	22	2.1	±0.1	Urine	(Slaunwhite)
	—	2.1	—	Pure compound	(Engel, 1950)

conditions employed, the reaction appears to favor the accumulation of the later compound. The nature of the solvent and the relative amount of substrate significantly affect this reaction (Tables 1 and 2).

The relative insolubility of estrone and estradiol in aqueous media has necessitated using organic solvents to disperse the hormones in the incubation mixture. It is possible that ethanol has an inhibitory effect on oxidative enzymatic reactions. Such an inhibition seems borne out by the comparison of results of incubation of estradiol and estrone with and without ethanol. Alcohol may inhibit the oxidative breakdown of estrone, allowing its accumulation without affecting the estrone-estradiol interconversion. The latter reaction is probably catalyzed by a dehydrogenase system (DeMeio *et al.*, 1948; Coppedge *et al.*, 1948), which is unaffected by ethanol in the concentrations used. Comparable total recoveries in the aqueous and alcohol experiments make solubility differences between the two solvents less likely as a factor.

The hormone concentration also appears to be a critical factor not only in the interconversion of estradiol and estrone, but in the further changes of these compounds. The apparent discrepancies in extents of estrogen "inactivation" (Heller, 1944) which have been reported by investigators using different hormone concentrations may be explained by the dependence of the reactions on hormone concentration. If a dehydrogenase system is involved in estrogen metabolism as has been suggested by DeMeio *et al.* (1948) and Coppedge *et al.* (1948) then the critical factor related to the amount of tissue present may be the associated concentration of diphosphopyridine nucleotide. This hypothesis is supported by the differential increase of estrone yield over estradiol recovery as the hormone concentration is decreased. In other words, as the hormone concentration is decreased by increasing the amount of tissue present, there is a differential increase of dehydrogenase over oxidative activity.

Although the conversion of estrone to estradiol has been demonstrated *in vivo* (Pincus and Pearlman, 1943), the *in vitro* data have not been conclusive. Heller (1940) "activated" estrone by incubation with rat uterus slices and with cyanide inhibited liver slices. He suggested that the increased biological activity was due to the conversion of estrone to estradiol. Schiller (1945) using partitioning methods, demonstrated biological activity in the non-ketonic fraction of the "weak" phenols after perfusion of estrone through rat liver. He considered this material to be estradiol. The data in Table 5 demonstrate the conversion of estrone to estradiol by rat liver.

No attempt has been made in the present study to investigate the question of conjugation of estrogens by the liver but past work (Heller, 1940; DeMeio *et al.*, 1948; Schiller, 1945) has indicated that hydrolysis does not increase recovery of estrogens measured biologically. The question of *in vitro* conjugation requires further study.

The problem of the site of conversion of estradiol or estrone to estriol has not been settled. Schiller (1945) reported the recovery of estrogenic activity in the "strong" phenolic fraction after perfusion of rat liver with estrone and estradiol but this observation has not been confirmed.

Metabolism of Estrogens by Cirrhotic Liver and Hepatoma: Cirrhotic liver and hepatoma nodules retain the capacity to metabolize estradiol to estrone. Microscopic sections of these tissues demonstrated them to be typical histologically, hence contamination with normal liver cannot be invoked to explain metabolic activity. It is apparent that these diseased tissues retain enzyme systems capable of oxidizing estradiol, albeit to a lesser degree than normal tissues. When the hormone concentration was decreased sufficiently (0.006), the differences between normal and diseased tissues diminished. Again it should be noted that these conclusions are based on a small series in which there was a high degree of variability. In one experiment, hepatoma did not effect the conversion of estrone to estradiol.

Anaerobic Experiments: When estradiol or estrone was incubated with rat liver slices in evacuated Thunberg flasks or in an atmosphere of nitrogen, interconversion was observed but the recovery of added steroid was much higher than in the aerobic experiments. This observation is in disagreement with DeMeio *et al.* (1948) who reported no "inactivation" in nitrogen. Actually, his data revealed 9 to 17% inactivation but this was, as he stated, within the limits of error of the bioassay method. On the other hand, Levy (1947) reported 30–50% inactivation under anaerobic conditions. The finding of metabolic change in nitrogen does not, however, discredit the theory of a DPN linked reaction since a coupled oxidation-reduction system could provide enough hydrogen acceptors to reoxidize the reduced DPN and change limited amounts of estradiol or estrone in the absence of oxygen. By analogy with anaerobic glycolysis, a reduction would tend to be favored under anaerobic conditions.

SUMMARY

1. Counter-current distribution and photofluorometry have been applied to the separation, characterization and quantitative estimation of estrone and estradiol after incubation with rat liver slices.

2. The interconversion of estrone and estradiol occurs under both aerobic and anaerobic conditions.

3. Ethanol has a slight inhibitory effect on the oxidative breakdown of estrone and estradiol.

4. The extent of conversion of estradiol to estrone by normal and cir-

rhotic rat livers and hepatoma nodules depends on the hormone concentration.

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THE INTERCONVERSION OF ESTRONE AND ESTRADIOL BY HUMAN TISSUE SLICES[1,2,3]

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STUDIES of estrogen metabolism in animals have limited application to man because of the different metabolic pathways in different species. For example, estriol has been recovered only from human sources; epiestradiol is a product of rabbit and equine metabolism and the naphtholic estrogens are peculiar to equine species (Pincus and Pearlman, 1943). Comparative studies of the *in vitro* metabolism of estrogens by human and animal tissues have not been extensive, but species differences have been noted (Twombly and Taylor, 1942; Szego and Samuels, 1943; Samuels and McCauley, 1946). The biochemical interrelationships between steroid hormones and cellular function is at present a matter of intense interest. In order to measure directly the metabolic activity of normal and neoplastic tissues of man, an investigation of estrogen metabolism in such tissues was undertaken. The data revealed that the interconversion of estrone and estradiol as well as the further degradation of both of these compounds can be accomplished by a variety of normal and diseased human tissues.

A previous report (Ryan and Engel, 1953) described the application of counter-current distribution and photofluorometry to the separation, characterization and quantitative estimation of estrogens after incubation with rat liver slices. These same procedures have now been applied to the study of the metabolism of estrone, estradiol and estriol by human tissues.

METHODS

Tissue Preparation: All tissues were operative specimens which were obtained under aseptic conditions and kept on ice for a few minutes until ready for incubation. Slices

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[3] This is publication #778 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

were cut on a Stadie-Riggs microtome to 0.5 mm. thickness, blotted dry with filter paper and weighed. Sections were made for histologic diagnosis. In all cases, control specimens of tissue were extracted and analyzed fluorometrically. No contaminating estrogens were found.

Hormone Preparation: The specifications of the estrone and estradiol, the buffer medium and the techniques of incubation, extraction and analysis have been described (Ryan and Engel, 1952). Ethanol solutions of the hormones were used throughout and the concentrations of the steroids in the incubations were 0.01 and 0.02 mg. of hormone /mg. tissue/ml. buffer. The amounts of hormone ranged from 450–1020 μg.

Estriol[5] was freed from a small amount of contaminating estrone (1.2%) by an eight transfer counter-current distribution in 50% MeOH/CCl_4. The stock solution was prepared in absolute ethanol at a concentration of 5 mg./ml.

TABLE 1. CONVERSION OF ESTRADIOL TO ESTRONE BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estradiol concentration*	% Estradiol recovered	% Estrone yield
Placenta	Term	Air	.01	33	38
Placenta	4 month	Air	.01	31	11
Ileum	Normal	Air	.01	35	11
Liver	Cholangitis	Air	.02	62	5.9
Liver	Cholangitis	Air	.02	51	4.6
Liver	Cholangitis	N_2	.02	60	2.1
Adrenal	Hyperplastic	Air	.02	56	2.3
Adrenal	Hyperplastic	N_2	.02	55	0.56
Adrenal	Cushing's	Air	.02	57	1.0
Breast	Pregnancy	Air	.01	43	2.3
Breast	Pregnancy	N_2	.01	61	3.1
Breast	Fibroadenoma	Air	.01	63	1.3
Breast	Fibroadenoma	N_2	.01	82	0.56
Breast	Adenocarcinoma	Air	.02	50	0.60
Endometrium	Hyperplastic	Air	.02	75	1.7
Endometrium	Hyperplastic	N_2	.02	61	0.92
Myometrium	Fibroid	Air	.02	54	0.66
Myometrium	Fibroid	N_2	.02	61	0.45
Testes	Normal	Air	.02	71	0.50

* Hormone concentration = mg. Estradiol/mg. tissue/ml. buffer.

RESULTS

The results are presented in Tables 1 to 3. All tissues studied were found to have the ability to metabolize estrogens, but there are differences in the types and extents of the reactions. Total recoveries varied from 42 to 92% depending on the hormone used, the tissue with which it was incubated and the gas phase under which the experiment was conducted. Metabolic activity was observed under anaerobic conditions. Similar findings have been reported for rat liver (Ryan and Engel, 1953). Tissues of the digestive, reproductive and endocrine systems of man can convert estradiol to estrone. The 18–58% of the hormones unaccounted for represents conversion to as yet unknown metabolites, in addition to loss during extraction and analysis. This latter loss has been found to be approximately 10% of the starting material (Ryan and Engel, 1953).

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[5] We wish to thank the Schering Corporation and Parke, Davis and Company for the gifts of the estriol samples.

Estriol was recovered almost quantitatively after incubation with testis and term placenta. Four month placenta gave a lower recovery of estriol (Table 3). In none of these three experiments was estrone or estradiol found after incubation of estriol.

TABLE 2. CONVERSION OF ESTRONE TO ESTRADIOL BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estrone concentration*	% Estrone recovered	% Estradiol yield
Testes	Normal	Air	.02	47	7.0
Placenta	4 month	Air	.01	64	1.4
Placenta	Term	Air	.01	59	0
Kidney	Hydronephrosis	Air	.02	65	0.69
Endometrium	Hyperplastic	Air	.02	62	0.51
Adrenal	Hyperplastic	Air	.02	43	0.46
Adrenal	Hyperplastic	N_2	.02	62	0
Breast	Pregnancy	Air	.01	72	0.34
Myometrium	Fibroid	Air	.02	80	0

* Hormone concentration = mg. Estrone/mg. tissue/ml. buffer.

TABLE 3. INCUBATION OF ESTRIOL WITH HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estriol concentration*	% Estriol recovered
Testes	Normal	Air	.02	92
Placenta	Term	Air	.01	92
Placenta	4 month	Air	.01	67

* Hormone concentration = mg. Estriol/mg. tissue/ml. buffer.

DISCUSSION

Except in the cases of the testes, term placenta and ileum, the tissues used were abnormal histologically and/or functionally, therefore, the estrogen metabolism of these tissues is not necessarily comparable with their normal counterparts.

Normal Tissue: The testis, in marked contrast to the other tissues studied, converts estrone to estradiol to a greater extent than the reverse reaction. A possible explanation for this phenomenon is that the enzyme systems ordinarily involved in testosterone synthesis from Δ^4-androstene-3,17-dione are accomplishing the estrone-estradiol conversion. Dorfman (1948), Clark and Kochakian (1947), and Kochakian *et al.* (1952) have shown an intimate relation between androstenedione and testosterone and suggested that androstenedione may be a precursor of testosterone. The reaction concerned is the reduction of the 17-ketone group of androstenedione to the 17β-hydroxyl group of testosterone. It is possible that the 17-ketone group of estrone is reduced to the 17-hydroxyl group of estradiol by the same enzyme system.

One incubation of estradiol with terminal ileum resulted in an 11% yield of estrone (Table 1). This yield is higher than that found with any tissue other than placenta.

Placental tissue has by far the greatest activity of all the tissues studied, 11 and 38% yields of estrone being realized with four month and term placental tissue respectively. However, the reduction of estrone to estradiol by these tissues is minimal (Tables 1 and 2).

Abnormal Tissues: In confirmation of the work of Twombly and Taylor (1942), Samuels and McCauley (1946) and more recently Tagnon *et al.* (1952), the degradation of estradiol by human liver slices has been demonstrated. In addition, it has been shown here that this tissue can oxidize estradiol to estrone as well as degrade both to unrecognizable metabolites (Table 1).

Endocrine System: Adrenal cortex slices can effect the interconversion of estrone and estradiol (Tables 1 and 2). This is not surprising in view of the intense activity of this tissue in steroidogenesis.

Slices of human pregnancy breast, mammary fibroadenoma and adenocarcinoma (grade iii) can convert estradiol to estrone with comparable facility. Hyperplastic endometrium and uterine fibroid tissues have a similar activity. This is in contrast to the findings of Szego and Samuels (1943) who reported no conversion of estrogens by endometrial tissue taken from a woman in the third month of pregnancy. In one experiment, kidney tissue converted estrone to estradiol.

Under the experimental conditions employed, estriol does not seem to be a significant metabolic conversion product of either estrone or estradiol in any of the tissues studied. Moreover, testis and term placenta do not appear to have the capacity to cause destruction of estriol; hence, if it were formed by these tissues in any significant quantity it should have been detectable. The techniques used permit the detection and characterization of a minimum of 5 μg. of estriol.

The demonstration that a wide variety of human tissues can metabolize estrogens requires that the concept of the liver as the only and/or chief site of estrogen metabolism be questioned. It appears that such organs as breast, uterus, small bowel, testis, adrenal cortex, kidney and placenta are capable of metabolizing significant amounts of estrone and estradiol, and thus must be included in any consideration of the over-all metabolism of these compounds.

SUMMARY

1. A variety of normal and abnormal human tissues have the capacity to

interconvert estrone and estradiol and to degrade both to unrecognizable metabolites.

2. No conversion of estrone or estradiol to estriol was observed.

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INHIBITION OF DEVELOPMENT OF DCA HYPERTENSION BY A NEW SYNTHETIC STEROID

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IT HAS been well established that desoxycorticosterone acetate (DCA) administered to normal rats or to rats sensitized by high sodium intake and/or uni-nephrectomy will produce renal and vascular pathology accompanied by arterial hypertension (Selye, Hall, and Rowley, 1943; Friedman, Polley, and Friedman, 1948; Masson, Corcoran, and Page, 1949). Although the etiology of DCA hypertension has not been explained in a manner satisfactory to all workers in the field, there have been searches for agents which will either reverse an established DCA hypertension or prevent the development of DCA hypertension in experimental animals, both because of the possible clinical implications and because the use of such agents would be helpful in research on mechanisms in vascular disease.

Agents which have been reported to be effective in preventing the development of DCA hypertension in rats are: *Lipo-Adrenal Cortex*,[1] estradiol[2] (Friedman and Friedman, 1950), cortisone acetate[2] (Friedman, Friedman, and Nakashima, 1950), and Phenergan (Guillemin and Fortier, 1951).

Agents which have been without inhibitory effect on development of DCA hypertension are Compound S acetate (Masson, Corcoran, and Page, 1950), progesterone, testosterone, hyaluronidase, "acetoxy-pregnenolene," "saturated 12-keto-DCA" (Friedman and Friedman, 1950), and methylandrostenediol (Skelton, 1952).

The present paper presents data on a new steroid, "U-0160," 3,21-diacetoxy-5,7,9, pregnatrien-20-one maleic anhydride adduct (Levin,

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[1] Registered Trade-Mark, The Upjohn Company, for cottonseed oil solution of hog adrenal extract.

[2] The authors cited do not feel that the anti-hypertensive action of estradiol and of cortisone acetate represent true antagonism to the "cardiovascular-renal effects of DCA" because of the debilitation of the animals receiving them. Cortisone, in their experiments, did not prevent the cardiac and renal enlargement produced by DCA. It did, however, partially prevent the DCA-induced rise in blood pressure. The effect of estradiol was termed "non-specific," although it alleviated the hypertensive effect of DCA very markedly.

Spero, McIntosh, Wesner, Meinzer, Searcy, and Thompson, 1951) which has the ability to prevent the blood pressure changes which accompany administration of DCA to a sodium-sensitized, uni-nephrectomized rat. The structure of this compound is shown in Figure 1.

METHODS AND MATERIALS

A. *Animals.* All experments were carried out on young male rats of the Upjohn strain (a colony developed from Sprague-Dawley stock), 120 to 140 grams body weight at the beginning of the experiment. The rats were unilaterally nephrectomized (left kidney) and were given a diet of Archer dog food and 1% sodium chloride drink. Beginning on the day following nephrectomy and continuing until the end of the experiment, all animals were injected with (A) 2 mg DCA, (B) 2 mg DCA plus 2 mg U-0160, (C) 2 or 4 mg U-0160, or (D) cottonseed oil only. All preparations were made so that the daily dose was contained in 0.2 ml cottonseed oil. This concentration exceeds the solubility of U-0160, and the resulting product was a finely-divided suspension.

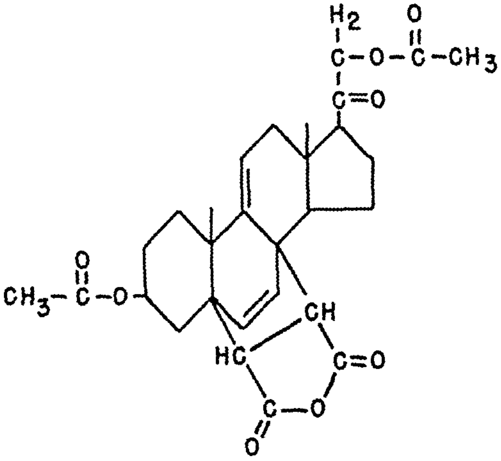

FIG. 1. Structural formula of U-0160.

All animals were injected once daily for 16 or 17 days. All rats' blood pressures were estimated three times during the experiments: The first two sets of determinations were taken to accustom the animals to the apparatus (see below for description of blood pressure estimation technique), and to trace the course of blood pressure changes. The definitive values, however, are those obtained on the seventeenth to eighteenth days, the day before sacrifice of the rats.

The rats were killed with chloroform and autopsied on the day following the last blood pressure estimation. The whole animal, the cardiac ventricles, the kidney, and, in some animals, the adrenal and thymus glands were weighed. Careful attention was given to the presence or absence of gross lung lesions, as it has been found that the occurrence of infected lungs is associated with highly aberrant blood pressures.

All experiments were designed with five or six animals in a single dosage group. Due to the occurrence of lung lesions in a number of animals and the necessity for discarding the contributions of these animals to the data, the results reported below include a number of groups of three or four rats.

Essentially the same experiment was performed at three widely separated times, as described with the results presented below.

B. *Blood Pressure Estimations.* The blood pressure estimations were carried out on conscious, unrestrained rats by a method involving measurement of optical density of the right hind foot during the deflation of a pneumatic cuff wrapped around the lower leg. The apparatus was constructed in The Upjohn Company Department of Physics by Mr. C. D. Alway and Mr. D. C. Cummings, incorporating principles described by two other laboratories: Kersten, Brosine, Ablondi, and SubbaRow (1947) found that a characteristic change in light transmittance of the rat foot occurred when the descending external pressure equalled the systolic blood pressure; Olmsted, Corcoran, Glasser, and Page (1948) described an apparatus which provided simultaneous recordings of the cuff pressure and the volume of the foot measured with a strain gauge.

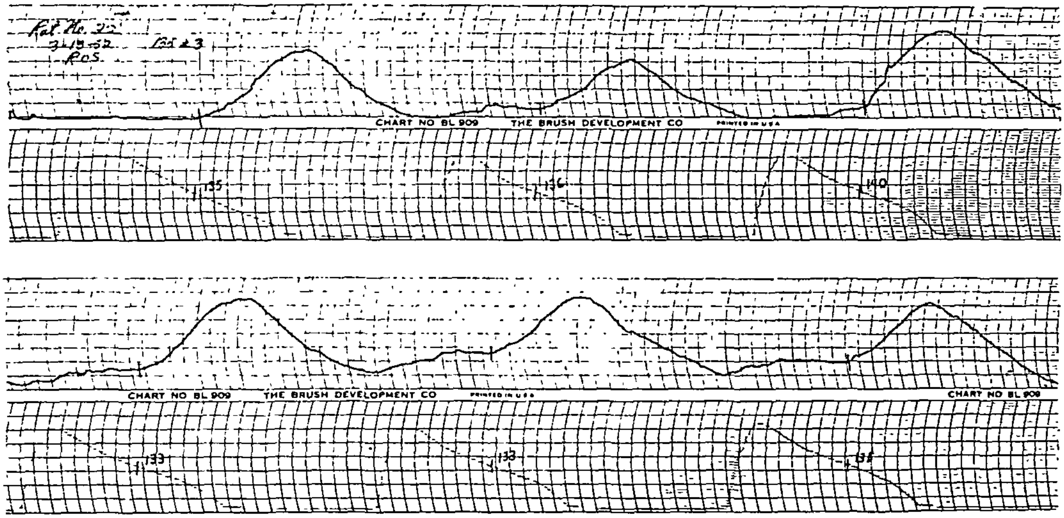

FIG. 2. Photograph of a record of blood pressure estimation with the Upjohn photoelectric tensometer. The record has been cut in half for conservation of space. The lower line indicates air pressure in the cuff, the upper line indicates photocell current. The point at which the upper line changes direction sharply is indicative of blood flow into the foot.

Our apparatus is constructed so that an intermittent light source of a specific frequency is transmitted through the foot of the lightly-held rat and impinges upon a photoelectric cell. The photo-cell current, routed through a series of amplifiers, activates the pen of a Brush Development Co. recorder. The pneumatic pressure in the bulb-cuff system is reflected by the expansion and contraction of a bellows which, through a rack-and-pinion mechanism, activates a potentiometer connected with a second pen on the Brush recorder.

The blood pressure work was all carried out in an insulated "quiet room" with extraneous noise reduced to a low level. The rats were handled as gently as possible and were placed in the rat holder in a manner designed for comfort and freedom from excessive restraint. After the first few determinations the animals were generally cooperative.

The variation in results obtained by the method is fairly large. We, therefore, rou-

TABLE 1. BLOOD PRESSURE ESTIMATIONS

Date	Treatment[2]	No. rats	Systolic blood pressures in mm Hg.[1]		
			I	II	III
4-51			*Days 5 and 6*	*Days 11 and 12*	*Day 18*
	Control[3]	6	129 (114–144)	129 (120–139)	136 (128–144)
	DCA	6	142 (118–166)	156 (138–184)	173 (153–193)
	DCA plus U-0160	6	119 (102–137)	122 (99–151)	140 (135–150)
	U-0160	6	138 (128–157)	128 (115–140)	125 (102–153)
6–51			*Days 10 and 11*	*Day 14*	*Day 17*
	Control[3]	4	127 (118–138)	133 (119–150)	130 (126–135)
	DCA	5	160 (135–202)	167 (140–197)	171 (146–197)
	DCA plus 2 mg. U-0160	5	130 (105–154)	122(110–135)	129 (115–135)
	DCA plus 4 mg. U-0160	4	126 (109–160)	130 (122–143)	133 (121–146)
	U-0160[4]	5	147 (126–190)	128 (119–140)	140 (128–169)
3-52			*Days 2 and 3*	*Day 8*	*Days 17 and 18*
	Control[3]	3	118 (101–141)	137 (123–164)	133 (127–138)
	DCA	5	112 (100–125)	154 (133–169)	167 (152–186)
	DCA plus U-0160	4	114 (98–136)	113 (105–123)	129 (120–142)
	U-0160	5	129 (120–135)	118 (94–141)	120 (101–151)

[1] Figures in parentheses indicate extremes within groups. Days indicated are days after nephrectomy.
[2] All doses 2 mg. per rat per day unless otherwise indicated.
[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.
[4] Suspended in saline. All other preparations suspended in cottonseed oil.

TABLE 2. BODY AND ORGAN WEIGHTS[1]

Date	Treatment[2]	No. rats	Body weights	Cardiac weights	Kidney weights
4-51	Control[3]	6	179 (155–214)	324 (308–367)	705 (594– 968)
	DCA	6	160 (125–182)	442 (336–457)	823 (700– 920)
	DCA plus U-0160	6	174 (156–201)	380 (358–408)	779 (708– 860)
	U-0160	6	175 (155–218)	327 (319–324)	634 (542– 784)
6-51	Control[3]	4	171 (157–182)	308 (290–340)	640 (590– 690)
	DCA	5	187 (172–203)	379 (350–440)	882 (740–1100)
	DCA plus 2 mg. U-0160	5	131 (110–156)	336 (310–400)	974 (910–1060)
	DCA plus 4 mg. U-0160	4	151 (139–163)	355 (330–380)	1237 (1070–1380)
	U-0160[4]	5	175 (166–188)	340 (310–370)	738 (660– 910)
3-52	Control[3]	3	199 (174–220)	304 (293–320)	723 (690– 825)
	DCA	5	169 (150–190)	366 (350–405)	902 (800–1020)
	DCA plus U-0160	4	152 (128–188)	292 (202–352)	1044 (755–1185)
	U-0160	5	176 (166–188)	287 (271–298)	885 (668–1397)

[1] Organ weights expressed as mg. per 100 g. body weight. Figures in parentheses indicate extremes within groups.
[2] All doses 2 mg. per rat per day unless otherwise indicated.
[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.
[4] Suspended in saline. All other preparations suspended or dissolved in cottonseed oil.

Note that administration of U-0176 did not antagonize the effects of DCA on blood pressure to any significant extent, nor did the administration of 2 mg per day of maleic anhydride.

The increase in renal mass caused by U-0160 is interesting, and may play a role in the blood pressure effects produced by this compound. We have insufficient data to attempt to explain the renal effects of U-0160 because the kidneys of these animals are undergoing changes which are the resultant of several factors: compensatory hypertrophy as a result of removal of the contralateral kidney, response to the sodium-DCA-induced diuresis, and response to the renomegalic effect of DCA itself. The role of U-0160 in the structure and physiology of this type of kindey is, therefore, a research problem in itself.

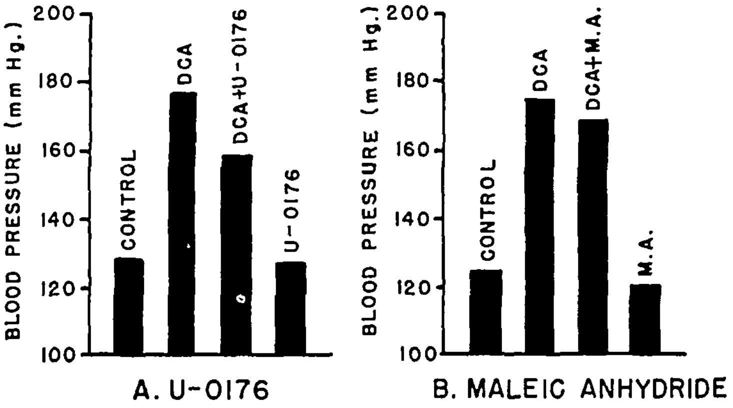

FIG. 4. A. Terminal blood pressures (Day 17–19) of rats receiving 2 mg DCA per day and/or 2 mg U-0176 per day; pooled data of two experiments similar in design to those shown in Figure 2. B. Terminal blood pressures (Day 19) of rats receiving 2 mg DCA per day and/or 2 mg maleic anhydride in aqueous solution. Single experiment, four or five rats per dose.

SUMMARY

U-0160 (3,21 diacetoxy-5,7,9-pregnatrien-20-one maleic anhydride adduct) was effective in preventing the hypertension produced by DCA in the unilaterally nephrectomized, sodium-sensitized rat. This property is not shared by U-0176 (U-0160 less maleic anhydride) or by maleic anhydride itself.

U-0160 also largely prevents the cardiac hypertrophy accompanying DCA hypertension, but augments the increase in renal mass.

It is considered improbable that the action of the compound is through release of 11-oxygenated hormones from the adrenal cortex, as there is no evidence of adrenal hypertrophy or thymic involution produced by the compound.

Acknowledgments

The authors are grateful to Dr. R. H. Levin, The Upjohn Company, Kalamazoo, Michigan, for generous supplies of U-0160 and U-0176 and for his criticism of this manuscript.

Thanks are expressed to Mr. L. C. Obermeyer who assisted with the blood pressure estimations.

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ASSAY OF ACTH ACTIVITY BASED ON THE EOSINOPENIC RESPONSE IN NORMAL AND HYPOPHYSECTOMIZED MICE[1]

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SINCE 1937, a number of assay procedures for ACTH have been proposed. These have generally been based upon a change in size of the adrenal glands of normal rats and baby chicks, or changes in the adrenal glands of hypophysectomized animals. The hypophysectomized rat has been the animal of choice, and the effect of ACTH on adrenal repair, maintenance, ascorbic acid or cholesterol levels have been used as end points in the assay. The ascorbic acid depletion test proposed by Sayers, *et al.* (1948) has proved to be the most satisfactory one. It is an extremely sensitive test, reasonably accurate and very useful in detecting small amounts of hormone in body fluids. (Gemzell, 1952.)

At the present time, the role of ascorbic acid in the synthesis or release of adrenal cortical hormones is unknown (Sayers, 1950). The factor in the ACTH that brings about the change in ascorbic acid level, may not be the same factor that produces an increase in size of the adrenal or release of the adrenal hormones. Reinhardt, *et al.* (1951a, b and c) compared the results of the ascorbic acid depletion test with those of the eosinopenia and adrenal maintenance tests. He found that the results do not compare favorably. A high ascorbic acid depleting power does not necessarily correspond to a high rate of secretion of the cortical hormones as measured by a reduction in eosinophils or in thymus and lymph node weights. These data have led a number of workers to suggest that in order to obtain accurate results in the assay of ACTH preparations several bioassay methods must be used (Greenspan, Li, Simpson and Evans, 1950; Selye, 1951).

In recent years a bioassay method for adrenal cortical hormones has been developed which is extremely sensitive, accurate and relatively easy to perform in mice (Speirs and Meyer, 1951; Speirs, Wragg, Bonner and Homburger, 1951). This assay is based upon the ability of the adrenal

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[1] This paper was presented before the meetings of the Endocrine Society on June 6, 1952. It is part of a project to study the activity of the adrenal cortex in health and disease and was supported by a grant from the New York Foundation.

cortical hormones to reduce the number of circulating eosinophils in adrenalectomized mice during a three hour period. It should be possible to modify this eosinophil method to assay ACTH. The assay would then measure the ability of ACTH to release the 11-oxycorticosteroid hormone from the adrenal cortex and would thus differ from the ascorbic acid depletion, repair or maintenance tests, and could be used to supplement them. Furthermore, it would be possible to measure the duration of the ACTH action upon the adrenal releasing the hormone. Such an assay might be very useful in developing and standardizing long-acting ACTH preparations.

The following paper is a report of a series of investigations to determine if the eosinopenic response of normal mice can be used to assay long acting ACTH preparations.

MATERIAL AND METHODS

The mice used in these experiments were the BBF_1 hybrid animals developed for the assay of adrenal cortical hormones; mice of strain 129 were used for comparison. The BBF_1 mice are the offspring of a cross between C57BR/cd and C57BL/6. Only male mice approximately 6 weeks of age and weighing between 20 and 25 grams were used.

The ACTH used in these experiments was supplied by Armour and Company, and assayed by them according to the ascorbic acid procedure. These hormones were used in order to determine possible assay procedures and no effort has been made to compare the eosinopenic values of various types of ACTH.

The techniques of performing eosinophil counts described earlier (Speirs and Meyer, 1949; Speirs, 1952a) have been modified slightly. Standard WBC diluting pipettes were used and the cells were counted in an Eosinophil Counting Slide (Speirs, 1952b) using a bifocal microscope containing 15× wide field oculars and 16 mm. objective. The eosinophil diluting fluid consisted of the following:

Phloxine B	0.02	gm.
Diethylene Glycol	2	ml.
Alconox (or similar detergent)	0.002	gm.
Distilled Water	85	ml.
Acetone, added just before using	15	ml.

Precautions were taken in order to avoid an eosinopenia due to stress. To avoid disturbances due to fighting, etc., the mice were kept in individual cages which were placed in a dark cabinet maintained at 27° C. for at least 24 hours prior to the experiment. Food, consisting of Purina Fox Chow with a weekly supplement of rolled oats, and water, were given ad libitum. Eosinophil counts were performed within 10 minutes after the animals were first disturbed. They were removed from their cages and placed in a battery jar containing wood chips and warmed by a 100 watt lamp. As soon as the animals became active, their tails were washed in warm soapy water, rinsed, and a small nick made through the skin and through one of the prominent blood vessels. Profuse bleeding occurred in all cases. The first two drops of blood were discarded, and fresh blood was drawn into the WBC pipette and diluted 1:20 with the above-mentioned diluent. One pipette of blood was taken from each animal, and from that two chambers on the Eosinophil Counting Slide were loaded. After allowing the cells to settle and stain for approximately 15 minutes, the eosinophils in both chambers were counted. The total count of both chambers (twenty $1 \times 1 \times 0.2$ mm. volumes) was multiplied by 5 to obtain the number of cells per cu. mm. of blood.

The changes in circulating eosinophils were given in this paper as percent decreases. They were computed as follows:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$$

All positive values (an increase in eosinophils) were recorded as zero percent decreases.

PROCEDURE AND RESULTS

In designing any assay procedure involving the fluctuation of eosinophils in normal animals, one must first consider the normal variation of these cells, particularly the diurnal variation (Halberg, 1951). In order to determine this in our animals, normal male mice were maintained in individual cages and kept in a dark cabinet with ample food and water. These mice were considered to be relatively undisturbed or free of stress prior to the experiment. At three hour intervals throughout the day and night, groups of animals were removed from their cages without disturbing the remaining animals, and eosinophil counts were performed. Each animal was used for only one eosinophil determination. This was repeated on two separate occasions and a total of 12 eosinophil counts were obtained for each period. The mean eosinophil counts and the standard error of the mean are plotted in Figure 1.

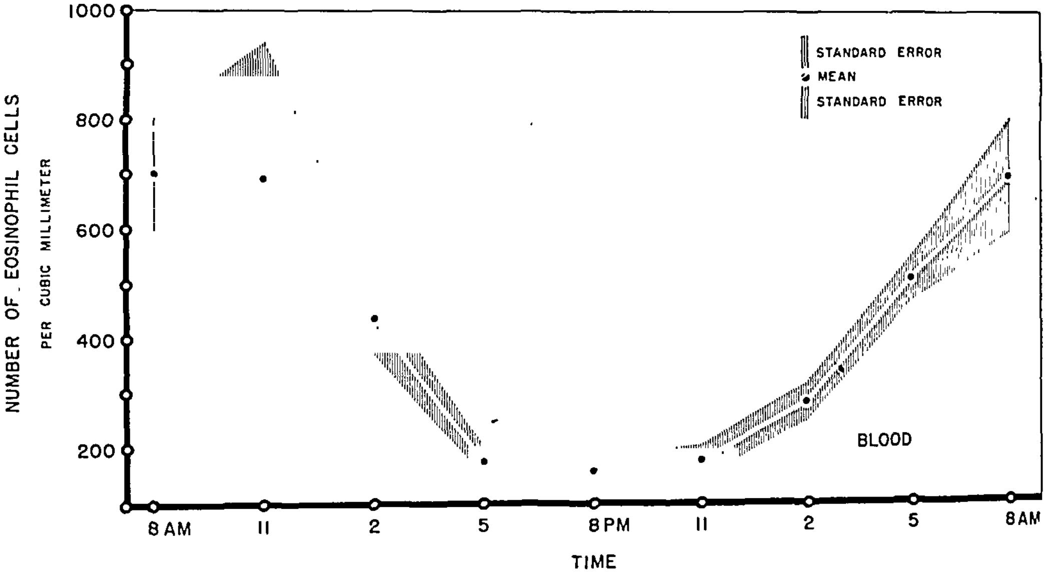

FIG. 1. Diurnal Variation of Eosinophil Cells in Blood. Each point represents the mean and standard error of 12 normal male BBF$_1$ mice.

It may be seen that the number of eosinophils fluctuates greatly during the day. A peak of 700 cells per cu. mm. was obtained at 8 and 11 A.M. and a low of 155 cells occurred between 5 and 11 P.M.

The next factor to consider is the effect of mild stress upon the number of circulating eosinophils. Figure 2 illustrates the mean and standard error of the number of eosinophils in normal C57 Brown mice following mild stress such as handling (from Speirs and Meyer, 1949). A similar eosinopenia was also obtained in the BBF_1 mice (unpublished data). Thus an eosinopenia occurs in these animals following mild stress. There is complete recovery within 24 hours after the animals are submitted to a single mild stress.

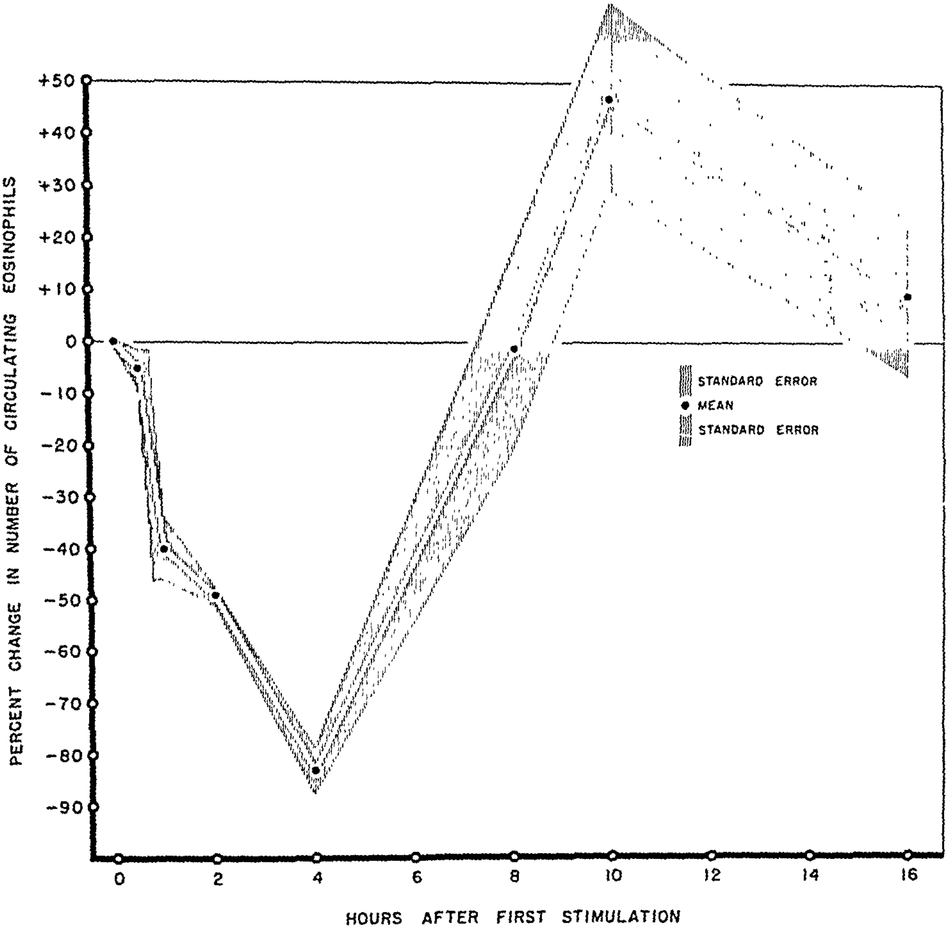

Fig. 2. Variation in the Number of Circulating Eosinophils in Normal Male C57 Brown Mice Following Mild Stress. Each point represents the mean and standard error of 9 mice.

Figure 3A shows the response curve of mice which had received subcutaneously 2 I.U. of ACTH in $\frac{1}{4}$ cc. of saline. This curve is essentially the same as the one obtained following a mild stress. By 10 hours recovery had begun and was complete by 18 to 24 hours.

Figure 3B presents results obtained following the subcutaneous injection of 2 I.U. of ACTH suspended in 0.1 cc. of gelatine. It may be seen that in the 6 and 10 hour groups, there was a decrease in eosinophils of 98% and 99%. Recovery began by 18 hours and was complete by 24 hours.

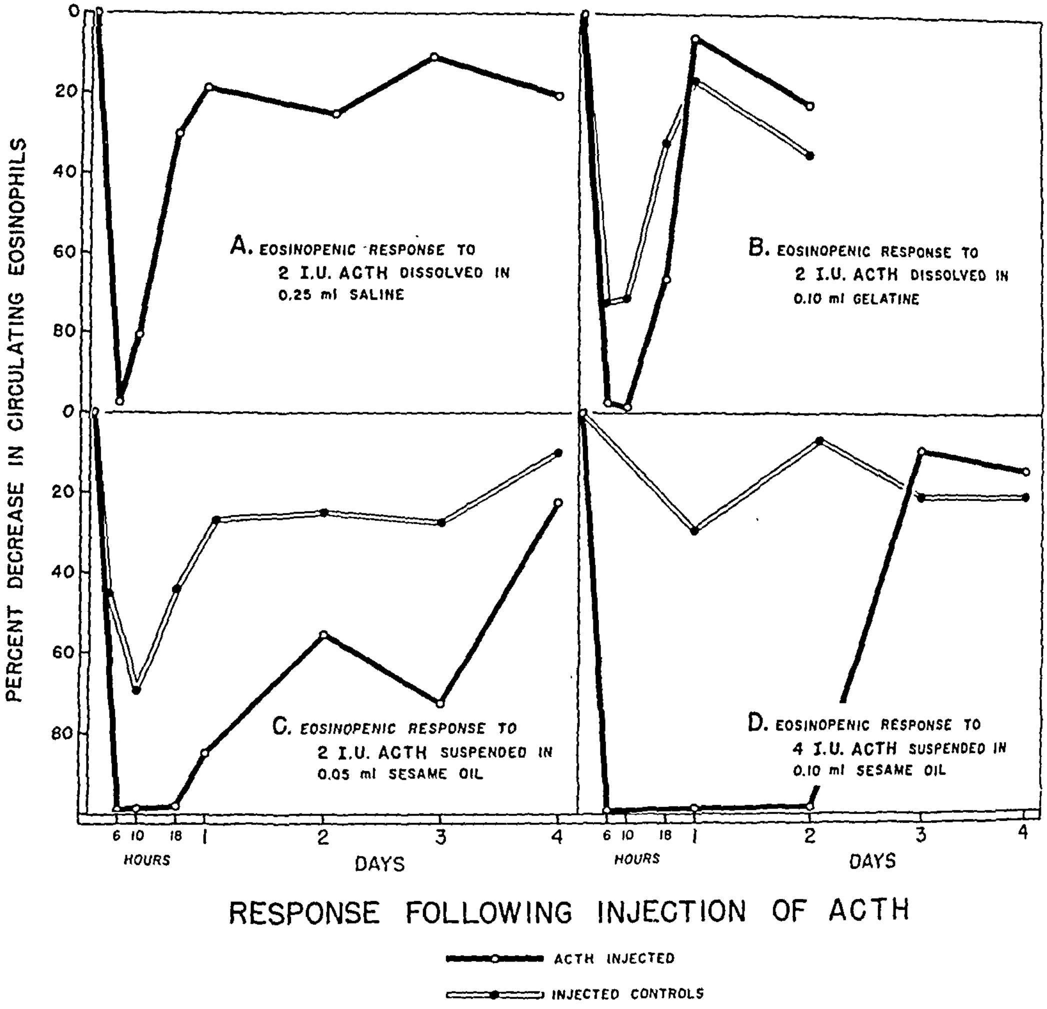

FIG. 3. Eosinopenic Response of Normal Male BBF$_1$ Mice to ACTH. Each point represents a mean obtained from at least 6 mice.

Figures 3C and D show animals which were injected with the ACTH suspended in sesame oil. In the group receiving 2 I.U., there was a complete absence of eosinophils 6 hours after the injection, and a 99% and 98% decrease in the 10 and 18 hour groups. Twenty-four hours following the injection an eosinopenia of 85% was found. Recovery began by 48 hours. In the group receiving 4 I.U. of ACTH suspended in sesame oil recovery did not occur until 72 hours following the injection.

It should be noted that in all the animals which received control injections, complete recovery in the number of circulating eosinophils occurred within 24 hours. This agrees with earlier published data in which the effects of epinephrine and mild stress were studied (Speirs and Meyer, 1949). However, the ACTH suspensions in gelatine and oil produced an eosinopenia which persisted for 24 hours or more. These data suggested that possibly an assay for ACTH could be developed in which only the 24 hour response was considered.

TABLE 1. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH DISSOLVED IN GELATINE

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS
0.25 ml.	0.5	6	50 ± 11.1
	1.0	6	59 ± 6.5
	2.0	5	95 ± 2.1
	3.0	6	97 ± 1.3
	5.0	6	99 ± 0.4
0.5 ml	0.5	6	58 ± 11.8
	1.0	6	55 ± 9.5
	2.0	6	96 ± 1.0
	4.0	5	100 ± 0.4
	10.0	6	100 ± 0
0.1 ml	2.0	12	6 ± 3.1
0.25 ml	2.0	5	95 ± 2.1
0.5 ml	2.0	6	96 ± 1.0
0.25 ml	0	6	25 ± 9.3 CONTROLS
0.5 ml	0	6	51 ± 13.6 CONTROLS

Table 1 summarizes the data in a group of 76 mice which had received various gelatine suspensions of ACTH. In the first group the ACTH was suspended in 0.25 ml. of gelatine. It may be seen that 0.5 I.U. of ACTH produced an average eosinopenia of 50%, 24 hours after the injection. One I.U. produced a slightly greater response, and 2, 3 and 5 I.U. produced 95%, 97%, 99% decreases respectively. The same responses were obtained when the ACTH was suspended in 0.5 ml. of gelatine. However, if the volume of gelatine was reduced to 0.1 ml., no significant eosinopenia occurred. The animals which had received 0.25 and 0.5 ml. showed an average fall in eosinophils of 25% and 51% respectively. It appears that the larger quantities of gelatine (0.5 ml.) produce a stress reaction which lasts throughout the 24 hour period.

Table 2 shows results obtained following the injection into 120 mice of ACTH suspended in sesame oil. It may be seen that a graded dose response is obtained when the ACTH is suspended in 0.05 ml. of oil. Similar results were obtained when a volume of 0.1 ml. was injected, but as the volume was increased to 0.2 and 0.4 ml. responses became less pronounced. The control mice which had received 0.4 ml. of oil had an eosinopenia equal to the group that had received the same volume containing 2 I.U. ACTH. It would appear from the data presented here that the smaller volume of the vehicle produced the most consistently high response.

TABLE 2. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH SUSPENDED IN SESAME OIL

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS	
0.05 ml.	0.25	4	6 ± 6.5	
	0.5	4	13 ± 13.5	
	1.0	10	44 ± 9.2	
	2.0	12	92 ± 3.4	
0.1 ml.	0.25	9	6 ± 5.6	
	0.5	9	28 ± 9.2	
	1.0	12	30 ± 11.5	
	2.0	8	86 ± 12.5	
	4.0	9	89 ± 11.1	
0.05 ml.	2.0	12	92 ± 3.4	
0.10 ml.	2.0	8	86 ± 12.5	
0.20 ml.	2.0	6	68 ± 12.9	
0.40 ml.	2.0	6	48 ± 18.1	
0.05 ml.	0	6	▨ 31 ± 8.2	CONTROLS
0.1 ml.	0	6	▨ 29 ± 10.5	
0.2 ml.	0	6	▨ 11 ± 6.6	
0.4 ml.	0	3	▨ 49 ± 24.9	

Table 3 shows the statistical treatment of the data obtained when two strains of mice were injected with 1 and 2 I.U. of ACTH suspended in $\frac{1}{4}$ ml. of gelatine. The first line shows results obtained when two groups of BBF_1 mice were injected with ACTH. The theoretical potency was 100%, the potency ratio obtained was 98.9%, with a standard error of 30%. The index of precision was 0.33 for the 6 sets, indicating a reasonably high degree of accuracy. In line 2 are the results obtained when 11 pairs of mice from each of two strains are compared. In this case the sensitivity ratio was 96% with a standard error of 11%. The index of precision was 0.16, indicating a very high degree of accuracy.

Injections of ACTH and gelatine were made into mice at periods varying from 4 to 30 days after hypophysectomy. A regression formula of $Y = 68 + 67.9 \text{ Log } X$ was obtained for 21 mice injected with $\frac{1}{2}$, 1 and 2 I.U. of ACTH in gelatine. Approximately the same sensitivity was obtained as in normal mice.

TABLE 3. STATISTICAL RESULTS OBTAINED WHEN STRAIN BBF_1 AND STRAIN 129 MICE ARE INJECTED WITH 1 AND 2 I.U. ACTH IN 0.25 ML. GELATINE

COMPARISON OF BBF_1 AND 129 MICE FOR ASSAY OF ACTH

MICE		RATIO OF	b				
COMPARISON	N	DOSES OF ACTH I.U.	SLOPE	S D	λ	POTENCY RATIO ± S E %	t SLOPE
BBF_1 vs BBF_1	6	$\frac{2}{1}$	− 160	53.3	0.33	98 ± 30	.13
BBF_1 vs 129	11	$\frac{2}{1}$	− 121	20.9	0.16	96 ± 11	.56

Injections of 2 mg. growth hormone in gelatine or sesame oil did not produce a significant eosinopenia over a 24 hour period. On the other hand injections of very toxic oils such as turpentine rsulted in an eosinopenia which persisted for 24 hours or longer. This eosinopenia occurred even in the hypophysectomized animals. For example, injections of 0.05 ml. of turpentine into 7 hypophysectomized animals resulted in the death of 4 animals and an average eosinophil decrease of 76% in the remaining 3.

DISCUSSION

In this paper an attempt has been made to determine the various factors which would be involved in the assay of ACTH in intact mice. Earlier experiments had indicated that a decrease in the number of circulating eosinophils in a relatively short period of 3 or 4 hours was a specific response to the 11-oxycorticosteroids. An assay which is based upon the decrease in eosinophils following an ACTH injection would therefore measure the amount of 11-oxycorticosteroid hormone released from the adrenal cortex. However, there are a number of factors which make the 4 hour assay impractical in the mouse. Intact animals which have been subjected to handling or mild stress will show an eosinopenia even without the ACTH injections. Furthermore there is a great diurnal variation which must be taken into account. Animals which have been hypophysectomized will also respond to handling and epinephrine injections with a slight eosinopenia (Speirs and Meyer, 1949). In addition it is very difficult to obtain two blood samples from these animals within a period of 4 hours.

However, it was noted in the experiments reported in this paper that following mild stress or injections of non-toxic solvents the eosinophils would completely recover by 16 to 20 hours. The diurnal variation was also found to be consistent. On the other hand, injections of ACTH suspended in gelatine and oil produced an eosinopenia which persisted for 24 hours or longer. These factors suggested that an assay could be devised which would measure the ability of ACTH to depress the number of circulating eosinophils during a 24 hour period.

Injections of 0.5, 1 and 2 I.U. of ACTH were made into intact mice and the following regressions were obtained:

$$Y = 56.02 + 138 \text{ Log } X \text{ (in 0.25 ml. gelatine)}$$
$$Y = 51.1 + 133 \text{ Log } X \text{ (in 0.05 ml. sesame oil).}$$

Determinations of the accuracy of the method were also made (Table 3). These analyses indicated that the assay was reasonably accurate and repeatable with as few as 6 sets of animals. Further comparisons were made between the BBF_1 and the 129 strains. The first strain is known to be highly sensitive to small amounts of 11-oxycorticosteroids and, when adrenalectomized, has been found to be the most satisfactory strain to use for the assay of corticoids. BBF_1 animals are approximately 12 times more sensitive to cortisone acetate than the 129 mice. On the other hand, the 129 strain has been shown to be less responsive to mild stress and handling as measured by a decrease in the circulating eosinophils. It was an entirely unexpected observation that these two strains responded in a similar manner to the long acting ACTH. The 129 mouse is much more docile and easier to handle than the BBF_1 and would therefore make a better assay animal. Other strains are being investigated to determine their sensitivity to ACTH and 11-oxycorticosteroids.

The assay of ACTH by the eosinophil procedure as described above is far less sensitive than the ascorbic acid depletion procedure of Sayers (1948). However, it has the distinct advantage of determining qauntitatively the amount of release of the 11-oxycorticosteroids from the adrenal and the approximate period of time over which the release occurs. For example, very high doses of ACTH in water solutions have little or no eosinopenic action 24 hours after injection. However, if the ACTH is suspended in gelatine or sesame oil a marked eosinopenia occurs. An assay based upon the duration of eosinopenia would thus be very useful in standardizing and comparing long acting preparations.

Preliminary experiments have indicated that hypophysectomized mice may also be used to assay ACTH solutions. These animals appear to be as sensitive to the ACTH as intact animals. The primary difficulty with this assay in intact animals is that very toxic materials may produce an eosinopenia which persists for 24 hours or longer. Injections of large volumes of slightly toxic materials which are slowly absorbed might also produce a persistent eosinopenia even in hypophysectomized mice. Therefore, care should be taken to run simultaneous controls using identical type and quantity of vehicle.

SUMMARY

The eosinophils in the circulating blood of intact, undisturbed mice were found to have a marked diurnal variation. When counts were performed on groups of 12 mice, an average of 700 eosinophils per cu. mm. was obtained between 8 and 11 A.M. with a rapid decrease to a low value of 155 cells per cu. mm. by 8 P.M. Complete recovery occurred by 8 A.M. the following morning.

Mild stress produced a marked eosinopenia in 3 to 6 hours followed by an eosinophilia. There was a recovery in the level of circulating eosinophils within 24 hours.

Injections of ACTH in saline solution produced an eosinopenia which was almost identical to that produced by mild stress. However, when the ACTH was suspended in gelatine or sesame oil, the period of eosinopenia was prolonged up to 24 hours or more.

These observations suggested that an assay for ACTH could be performed in either intact or hypophysectomized mice. The per cent decrease in eosinophils over a 24 hour period was found to be proportional to the quantity of ACTH injected. Statistical analysis of the data indicated that the regression was $Y = 56.02 + 138\ X$ when 0.5, 1 and 2 I.U. of ACTH was suspended in 0.25 ml. gelatine and injected into intact mice. The index of precision of 6 sets of animals injected with 1 and 2 I.U. ACTH in gelatine was found to be 0.33.

Two strains of mice tested for this assay, the BBF_1 hybrids and the 129 mice, were found to give almost identical responses. The index of precision of 11 sets of animals was found to be 0.16.

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THE ACTION OF DESOXYCORTICOSTERONE ACETATE ON THE MAMMARY GLAND OF THE IMMATURE OVARIECTOMIZED RAT

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DESOXYCORTICOSTERONE acetate (DCA) exerts a progesterone-like action on the uterus (Van Heuverswyn, *et al.*, 1939a; Engle, 1941; Masson, 1943) and mammary gland (Mixner and Turner, 1942; Trentin and Turner, 1948; Speert, 1940) of several laboratory animals. It also shows estrogen-like activity on the mammary gland (Van Heuverswyn, *et al.*, 1939b; Leonard and Reece, 1942.) However, no comprehensive study of its action on the mammary gland of the rat has been undertaken. The experiments reported here were designed to elucidate the action of DCA on the mammary gland of the immature ovariectomized rat, alone, and in combination with estradiol or progesterone.

MATERIALS AND METHODS

Twenty-four day-old female albino rats were ovariectomized. Treatment was begun on the 29th day with daily subcutaneous injections of the hormones in sesame oil with the exception of the highest dose of DCA which was administered in propylene glycol.[3] A standard daily dose of 0.1 γg. estradiol and of 4.0 mg. progesterone was injected. The doses of DCA were varied from 1.0 to 10.0 mg. daily. The rats were treated for 12 days. Autopsy was performed the day after the last injection. The right abdominal-inguinal mammary glands of each animal were removed, fixed in Bouin's solution, stained with Mayer's haematoxylin, and stored in methyl salicylate for later study as whole mounts.

The areas of the glands were traced onto weighing paper and later measured with a Keuffel and Esser planimeter. The glands were trimmed of peripheral fat and connective tissue and weighed on a triple-beam balance. Lobulo-alveolar growth was rated on a similar scale. The uteri were also trimmed, split, blotted on bibulous paper, and weighed for comparative study.

RESULTS

Daily doses of 2 and 5 mg. DCA resulted in glands with ducts which were dilated and extended, and buds which were expanded (Table 1). There was no alveolar formation. Mammary gland areas were somewhat smaller

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[3] The steroids used in these studies were generously supplied by the Schering Corporation, Bloomfield, New Jersey.

than in the controls. The glands resembled histologically those of animals treated with estradiol alone. At a daily dose of 10 mg., DCA had no effect on mammary duct growth. The glands resembled those of untreated castrated controls, in spite of the fact that uterine growth was almost as great as that of rats treated with estradiol.

TABLE 1. INFLUENCE OF DCA OR ESTROGEN ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
None	5	64	105	6.0 ± 0.37	0.29 ± 0.070	0/5	32.2
0.1 γg. Estrogen	4	63	102	6.1 ± 0.35	0.36 ± 0.050	0/4	128.5
2 mg. DCA	3	65	110	5.0 ± 0.47	0.24 ± 0.015	0/3	Atrophic
5 mg. DCA	3	69	113	3.9 ± 0.32	0.26 ± 0.040	0/3	Atrophic
10 mg. DCA	2	50	93	5.9	0.36	0/2	93.5

Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

It is well known that estradiol and progesterone synergize to induce lobulo-alveolar growth. Since DCA apparently exhibited estrogenic properties in the preceding experiments, further experiments were designed to determine whether DCA would substitute for estradiol in this synergistic combination (Table 2). Animals injected with 4.0 mg. of progesterone daily showed glands with some duct and end bud expansion, and dubious lobulation. In all rats receiving 0.1 γg. estradiol and 4.0 mg. progesterone daily, the ducts and end buds were expanded, and excellent lobulation occurred.

TABLE 2. INFLUENCE OF PROGESTERONE, DCA AND PROGESTERONE, AND ESTROGEN AND PROGESTERONE ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
4 mg. Progesterone	3	71	114	7.8 ± 0.39	0.36 ± 0.048	1/3	53.3
4 mg. Progesterone + 2 mg. DCA	3	54	92	6.8 ± 0.58	0.28 ± 0.011	0/3	45.0
4 mg. Progesterone + 5 mg. DCA	3	62	106	6.3 ± 0.43	0.31 ± 0.020	0/3	84.2
4 mg. Progesterone + 0.1 γg. Estrogen	3	64	101	7.2 ± 0.67	0.35 ± 0.057	3/3	114.4

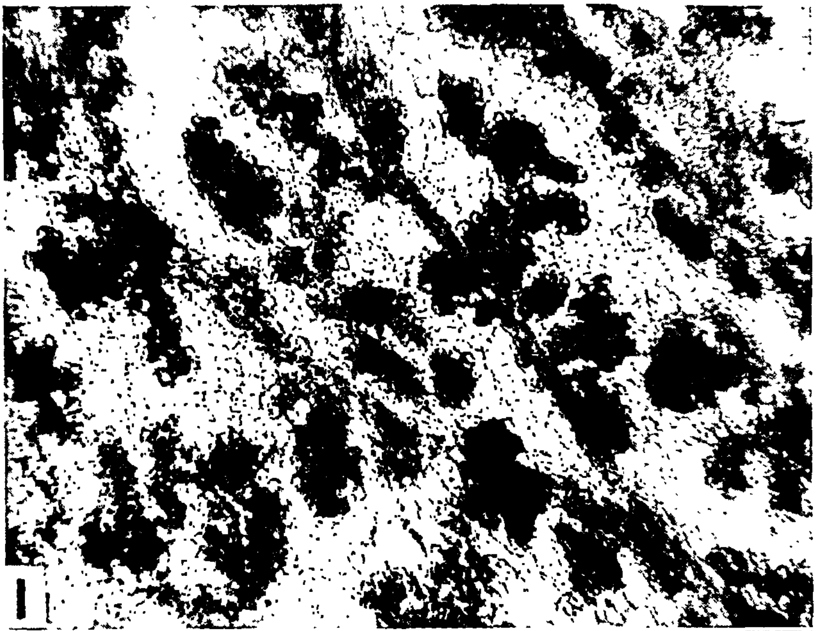
(Fig. 1). A combination of 4.0 mg. progesterone and 2 mg. DCA daily led to expanded ducts and buds, and, in two out of three rats, doubtful alveolar development. With a combination of 4.0 mg. progesterone and 5 mg. DCA daily, there was spotty duct extension and dilation, slight bud expansion, and no lobulation.

Rats given 5 mg. of DCA daily, in addition to progesterone, had uterine weights which were around 50% above rats receiving progesterone alone. Two mg. DCA failed to effect this increase. In rats treated with estradiol and progesterone, average uterine weight was more than 100% increased over the progesterone controls.

The next step was to determine whether or not DCA would exhibit any progesterone-like activity in combination with estradiol. The possibility of any additive effects with the estrogen was also looked for. Reference to Table 3 shows that 0.1 γg. estradiol and 2.0 mg. DCA daily did not elicit any lobulo-alveolar growth, while 0.1γg. estradiol and 5.0 mg. DCA led to some lobulation in four of five rats: three gave a "plus one" reaction, and one gave a "plus two" reaction. Mammary areas showed little change from those of rats receiving estrogen alone (Table 1). Estradiol, 0.1 γg., and DCA, 10 mg., daily led to a "plus three" in one and a spotty "plus two" reaction in two of five rats. One of the better responses is seen in Figure 2, comparable to that obtained with 0.1 γg. estradiol and 4.0 mg. progesterone (Fig. 1). Mammary areas were increased in this group, comparable to those of rats treated with estradiol and 4.0 mg. progesterone (compare with Table 2). Propylene glycol alone had no effect on the mammary glands, which were identical in appearance with those of the ovariectomized, uninjected rats. It is evident from these results that DCA at a dose of 5 mg. daily exhibits only a slight progesterone-like activity in the syner-

TABLE 3. INFLUENCE OF ESTROGEN-DCA COMBINATIONS ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Mammary gland			
		Change	Final	Area (cm.^{2})	Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen + 2 mg. DCA	4	55	94	5.1 ±0.44	0.34 ±0.041	0/4	120.3
0.1 γg. Estrogen + 5 mg. DCA	5	62	114	6.2 ±0.18	0.33 ±0.059	4/5	111.1
0.1 γg. Estrogen + 10 mg. DCA	5	42	92	7.5 ±0.63	0.31 ±0.029	2–3/5	111.3
Propylene glycol	2	—	—	Castrate Atrophy			—



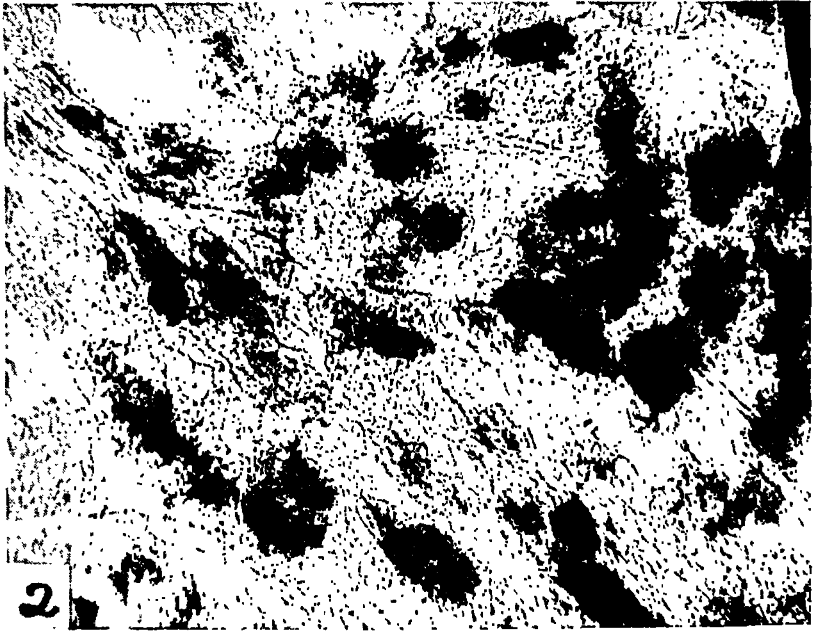


Fig. 1. Mammary gland of rat injected with 0.1 γg. estradiol and 4.0 mg. progesterone for 12 days.

Fig. 2. Mammary gland of rat injected with 0.1 γg. estradiol and 10.0 mg. DCA for 12 days.

gistic reaction with estradiol causing lobulation. Doubling the daily dose of DCA produced little additional effect on mammary lobulation. Nor could any additive effects with the estrogen be detected, either on the mammary duct system or on uterine weight.

The possibility remained that DCA, because of structural similarity to progesterone, might exhibit an additive or antagonistic action to the lobulo-alveolar induction by 4.0 mg. progesterone and 0.1 γg. estradiol daily. DCA, in doses of 1.0, 2.0, and 4.0 mg. daily failed to exert any additive or antagonistic effects, either on lobulo-alveolar growth, duct structure, or total area (compare results in Table 4 with those in Table 2). Uterine weights were unaltered by the superimposed DCA treatment.

TABLE 4. INFLUENCE OF DCA ON MAMMARY GROWTH ELICITED BY ESTROGEN AND PROGESTERONE

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen+ 4 mg. Progesterone +2 mg. DCA	2	77	116	6.9	0.33	2/2	111.7
0.1 γg. Estrogen+ 4 mg. Progesterone +2 mg. DCA	4	64	106	7.2±0.81	0.37±0.054	4/4	102.2
0.1 γg. Estrogen+ 4 mg. Progesterone +4 mg. DCA	4	58	101	6.8±0.60	0.33±0.038	4/4	114

DISCUSSION

DCA has mammary duct-stimulating activity in the mouse in doses of 0.5 mg. every other day (Van Heuverswyn, *et al.*, 1939b) and in the partially-hypophysectomized rat (Leonard and Reece, 1942). The present experiments furnish evidence that it exerts both an estrogen-like and a progesterone-like action on the mammary gland of the immature ovariectomized rat. Even though DCA is estrogenic when administered alone, it fails to substitute for estradiol in the synergistic reaction with progesterone to bring about lobulo-alveolar development. In this respect, its estrogenic activity is peculiar, and perhaps, atypical.

The amount of DCA required to produce even slight lobulo-alveolar growth in the rat is much greater than that reported by Mixner and Turner (1942) to produce lobulo-alveolar growth in the mouse. In their experiments, DCA was approximately one-third as potent as progesterone. An exact ratio of DCA to progesterone cannot be determined from the present data. However, it must be pointed out that the amount of progesterone, injected with estrogens, required to produce optimal lobulation is also greater in the rat (Curtiss, 1949, Smith, 1952, in preparation).

The progestin-like activity of DCA in these experiments may be due in part to a conversion of the injected DCA to progesterone, similar to that which has been reported for the human and chimpanzee (Horwitt, *et al.*, 1944), macaque (Zarrow, *et al.*, 1950), rabbit (Hoffman, *et al.*, 1943) and mouse (Hooker and Forbes, 1949). However, this conversion, or other metabolic pathways of DCA have not been demonstrated in the rat. Consequently, we have no hint as to what extent DCA itself or the metabolites of DCA are responsible for the mammotropic effects seen in the present experiments.

Other lines of evidence have implicated the adrenal cortex in mammary growth processes. These are the several reports that adrenalectomy affects mammary growth (Butcher, 1939; Reeder and Leonard, 1944; Trentin and Turner, 1947; Johnston and Smithcors, 1948; Cowie, 1949; Smith, 1951). In the last-mentioned study, lobulo-alveolar growth was unaffected by adrenalectomy, and the reduced duct response to the ovarian steroids and relaxin was restored by administration of DCA, or other corticoids. Other reported effects have varied widely, from slight stimulation to partial suppression of growth, but are not completely unreconcilable.

If the DCA-type compounds play more than a sustaining role for mammary growth in the rat, endogenous production must be enormous, and might even approach a toxic level. Three of nine rats receiving the highest dose of DCA, 10 mg. daily, died during the course of treatment, while rats receiving the propylene glycol alone remained healthy. These results suggest that this is not only a maximal hormonal dose, but also approaches a toxic pharmacological dose for this species.

It might be expected that since DCA resembles progesterone structurally, but does not itself exert the consistent lobulating activity of progesterone, it would interfere with the optimal growth elicited by estradiol and progesterone. The possibility that the doses of DCA were too low, or the dose of progesterone was relatively high needs further scrutiny. However, with the gravimetric ratios used: 1:4, 1:2, and 1:1, no evidence for any inhibitory effect was obtained. Nor was the reverse of this, an additive or synergistic effect, observed.

SUMMARY

Desoxycorticosterone acetate (DCA) alone exerts a duct-stimulating effect on the mammary gland of the immature ovariectomized rat. Alone, or in combination with progesterone, it does not effect any lobulo-alveolar growth. Five mg. of DCA daily, given simultaneously with estradiol, exhibits a slight lobulating action, but doubling the dose of DCA to 10 mg. produces little additional lobulation. No antagonistic or additive effect

of DCA upon the synergistic reaction of estradiol and progesterone in stimulating lobulation could be detected.

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EVIDENCE FOR A DUAL ACTION OF PARATHYROID EXTRACT IN MAINTAINING SERUM CALCIUM AND PHOSPHATE LEVELS[1]

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THE primary mechanism through which the parathyroid glands influence mammalian calcium and phosphate metabolism is still an unsettled problem. The results of past experiments have led to two divergent views regarding the action of the parathyroid hormone: one view maintaining that the principal role of the hormone is the regulation of the osteoblastic and osteoclastic activity of bone tissue, while the other view claims its action to be primarily in controlling the phosphate threshold of the kidney. For a recent review of the work the reader is referred to Greep (1948).

While much of the recent work has been in favor of the kidney threshold theory, evidence has also accumulated suggesting an extra-renal function of the hormone. Considerable work has been reported showing the influence of the parathyroids on bone metabolism both before and after removal of the kidneys (Selye, 1942; Ingalls *et al.*, 1943; and Barnicot, 1948). In addition, Monahan *et al.* (1944) produced a fall in serum calcium in dogs parathyroidectomized after total nephrectomy. Stoerk (1943) prevented a fall in serum calcium levels of rats after total nephrectomy and parathyroidectomy by administration of parathyroid extracts. Similarly, Stewart and Bowen (1951) produced a rise in blood calcium values after nephrectomy in both normal and parathyroidectomized dogs. On the other hand, Tweedy *et al.* (1947, 1950) failed to find any influence of this gland on radio-phosphorus and radio-calcium distribution in nephrectomized rats.

The purpose of this study is to present evidence that parathyroid extract may produce its primary influence on serum phosphate and calcium levels by independent and separate mechanisms.

MATERIALS AHD METHODS

More than 150 male Wistar rats, weighing between 200 and 250 grams were used in these experiments. The parathyroids were removed individually after the method of

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Richter and Birmingham (1941). Nephrectomies were performed in two stages, the left kidney being removed three to four days prior to the right. Since the rat is very resistant to the removal of the parathyroids, possibly due to the presence of accessory tissue, the test for hypoparathyroidism was a drop in serum calcium to below 7 mg.% and the appearance of a partial tetanic response in the animal. Animals not showing the external nervous manifestations were discarded.

Parathyroid extract[3] was administered subcutaneously in a water solution, each injection containing 50 USP units. Four injections were given over a 24 hour period and the animals were killed four to six hours after the last injection. Blood was removed by heart puncture prior to killing. Serum calcium levels were determined by the method of Clark and Collip (1934); serum phosphates as described by LePage (1949).

Except where otherwise indicated, the experimental procedure was as follows: On Day 1, the parathyroids were removed. The left kidney was removed either on the same or the following day. The animals were maintained on stock diet during the period of recuperation. The actual experiment began with the removal of food in the early evening of Day 4. Eighteen hours later, on Day 5, the parathyroidectomized animals which did not show external manifestations of parathyroid deficiency were discarded, and the remaining animals were divided into the various experimental groups. At this time the second kidney was removed and/or the first injection of parathyroid extract was given. Additional injections were given six and twelve hours later and on the morning of Day 6. The experimental procedures were terminated by bleeding the animals on the afternoon of Day 6. Control groups were included in each experiment and were treated identically in respect to food, removal of kidneys, etc.

RESULTS

The animals used in these experiments were divided into ten groups. Those in the first five groups had intact parathyroid glands, while the remaining animals were parathyroidectomized. A brief description of the experimental treatment of each group is given in Table 1, and the results are summarized in Table 2.

TABLE 1

Group	Parathyroid glands intact	Parathyroid glands removed	Kidneys removed		Parathyroid administration
			Left	Both	
1	*				
2	*		*		
3	*		*		*
4	*			*	
5	*			*	*
6†		*	*		
7		*	*		
8		*	*		*
9		*		*	
10		*		*	*

* For time sequence of operations and parathyroid extract administration see Materials and Methods.

† Killed at the time nephrectomy was completed in other animals; these serve as a baseline for further changes in serum and calcium values following complete nephrectomy.

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[3] Lilly's Solution of Parathyroid Extract.

TABLE 2. SERUM PHOSPHATE AND CALCIUM VALUES

Group	Calcium		Phosphate	
	No. of animals	Value ±S.E.	No. of animals	Value ±S.E.
A. *Intact parathyroids*				
1. Stock Animals	9	11.7 ±0.18	6	7.7 ±0.12
2. Controls	21	11.2 ±0.19	6	5.4 ±0.34
3. PTH*	7	12.1 ±0.40	7	5.0 ±0.31
4. Nephx†	11	11.6 ±0.32	7	14.6 ±0.61
5. Nephx-PTH	5	11.3 ±0.42	5	14.2 ±0.56
B. *Parathyroidectomized*				
6. PTX‡ only	21	5.7 ±0.19	4	15.3 ±1.12
7. Prenephx	8	6.7 ±0.31	7	10.6 ±0.43
8. PTH	8	10.1 ±0.57	8	6.3 ±0.41
9. Nephx	18	5.3 ±0.23	11	17.7 ±0.43
10. Nephx-PTH	22	7.8 ±0.25	16	17.4 ±0.44

* Injected with parathyroid extract.
† Nephrectomized.
‡ Parathyroidectomized.

COMPARISON OF SERUM CALCIUM VALUES

The average serum calcium value for the normal rat has been found by us to be over 11 mg.% (Group 1). In the animal with intact parathyroids, this value is maintained at least up to 42 hours of starvation (Group 2), and in the totally nephrectomized starved rats is still constant 36 hours after removal of the last kidney (Group 4).

The administration of parathyroid extract for 24 hours to the normal but starved rat produced a questionable rise in the serum calcium value (Group 3). Previous experiments had shown that if this dosage was continued for an additional two days it would, in normal fed rats, raise the serum calcium level to approximately 15 mg.%. As noted by Tweedy *et al.* (1950), no increase was seen in the serum calcium level of nephrectomized rats treated with the hormone for twenty-four hours (Group 5).

In the parathyroidectomized rat it was found necessary to starve the animal in order to produce a marked drop in the serum calcium. Up to six days after removal of the parathyroids, animals maintained on a normal diet usually had a serum calcium value in the low normal range and only rarely did this drop as low as 9 mg.%. However, if food is removed, as done in these experiments on Day 4, the calcium values drop rapidly and 18 hours later will be found to be approximately 6.7 mg.% (Group 7). 36 to 42 hours after removal of the food, the value will have dropped to 5.5 mg.% (Group 6). As seen in Group 9, nephrectomy does not appear to alter significantly this chain of events.

If, after starvation for 18 hours, parathyroid extract was administered to the parathyroid deficient rat, the serum calcium values rose rapidly and

24 hours later were found to be in the low normal range (Group 8). When total nephrectomy was completed prior to injection of the hormone, a rise in serum calcium was still produced though not as marked (Group 10). Thus, a further decrease was not only prevented, but an actual increase in the serum values was observed as shown by comparison of groups 7, 9, and 10.

COMPARISON OF SERUM PHOSPHATE VALUES

The serum phosphate level of normal animals has been found by us to be approximately 7.5 mg.% (Group 1). This value is, however, markedly affected by environmental conditions, particularly diet (Tepperman *et al.*, 1947). Starvation for 36 to 42 hours caused this value to drop to 5.4 mg.% (Group 2). Parathyroidectomy caused a rapid rise in the serum values. After the 18 hour starvation period, the phosphate level of those animals showing external manifestations of parathyroid deficiency had risen to approximately 10.6 (Group 6), and by 36–42 hours was up to 15.3 mg.% (Group 5).

Nephrectomy, in the normal animal, produced a rise in serum phosphate to approximately the same value as the non-nephrectomized parathyroid deficient animal (Group 4). Nephrectomy, however, increased only slightly the already high phosphate level of the parathyroidectomized rat (Group 9).

At the dosage used in these experiments, parathyroid extract treatment had no significant effect on the serum phosphate in the normal animal (Group 3). In the parathyroid deficient animal, the hormone caused a rapid drop in the serum values to a value approaching the normal condition (Group 8). Nephrectomy apparently negated this effect of the hormone entirely (Groups 9 and 10).

DISCUSSION

Since Collip *et al.* (1925) first discovered the influence of the parathyroids on calcium and phosphorus metabolism, it has become well-established that as a result of parathyroid removal the serum calcium level rapidly falls, accompanied by a corresponding increase in the serum phosphate value. Very little emphasis, however, has been placed on the observations, which have been shown before and confirmed by these studies, that the sequence of changes in the serum calcium levels is not influenced by total nephrectomy performed either at or subsequent to the removal of the parathyroids; and that only minor further increases in the phosphate serum values are produced by such removal of the kidneys.

It has also been well established that, in the hypoparathyroid animal in which low serum calcium and high phosphate levels are present, injection of parathyroid extract produces an immediate increase in the excretion of phosphate by the kidneys, accompanied by a lowering of the phosphate and raising of the calcium serum values. Removal of the kidneys prior or during this sequence of events has a marked influence only on the phosphate changes. All effect of the hormone on the phosphate levels seemingly is lost; however, the calcium values *will* continue to rise though the rate is somewhat restricted.

Careful consideration of these data and those presented by the investigators referred to in this work points very strongly to the facts that calcium and phosphate serum values can vary independently of each other; that normal calcium values can be maintained in the presence of phosphate values two and a half to three times their normal value, and that the effect of parathyroid extract on serum calcium of the parathyroid deficient rat is not negated by the removal of the kidneys.

In the light of these facts, one cannot see how any theory of the sequence of events produced by parathyroid extract can be tenable which makes renal excretion of phosphorus the only means by which the hormone can influence calcium metabolism, though indirect influences by means of changes in the phosphate levels very probably occur (Albright and Reifenstein, 1948).

At the present time there appears to be two alternatives: one which would conclude that the hormone exerts its influence directly on only one of these ions, and that the indirect influence on the other is caused, not only by the solubility product of these two ions in the blood, but also by some other extra-renal influence either in the bones or elsewhere; or that the hormone has a dual action, controlling phosphate primarily through its renal effect, and calcium primarily through an extra-renal influence.

Due to the wealth of data showing the influence of the parathyroids on renal excretion of phosphate and to the widely fluctuating serum phosphate levels possible in the presence of a constant calcium value, the latter alternative is considered to be more likely.

SUMMARY

The influence of nephrectomy upon the action of parathyroid extract has been studied in rats. This extract was administered to normal and parathyroid deficient rats which had been nephrectomized in a two-stage operation. Both serum phosphate and serum calcium values were determined. Nephrectomy appeared to nullify all effects of parathryoid extract upon serum phosphate levels. However, the effect of the extract on serum calcium, though somewhat suppressed, was still apparent. Not only did the parathyroid extract prevent a further decrease in serum calcium in the nephrectomized parathyroid-deficient rat, but when 200 USP units were

administered over a 24 hour period following removal of the last kidney, a definite rise in serum calcium was noted. Due to these observations, it is concluded that for its normal function, the parathyroid extract has a direct and independent effect on both calcium and phosphate metabolism.

ADDENDUM

Attention is called to the recent publication by Stewart, G. S. and H. F. Bowen (*Endocrinology 51:* 80. 1952) which appeared since submitting this manuscript. These authors are suggesting that the phosphate excretion factor of the parathyroid gland extract is an artifact, leaving the calcium mobilizing factor the primary function of the parathyroid glands.

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THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE RENAL TUBULAR REABSORPTION OF PHOSPHATE AND THE EXCRETION OF TITRATABLE ACID AND POTASSIUM IN DOGS[1]

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IT HAS been demonstrated that the renal tubules of dog and man are capable of reabsorbing a fixed and limited quantity of phosphate per unit of time when presented with an excess in the glomerular filtrate (Pitts *et al.*, 1944; Schiess *et al.*, 1948). Since absorptive capacity is independent of glomerular filtration rate over a fairly wide range (Ayer *et al.*, 1947) the renal mechanism which handles phosphate, like that which handles glucose, exhibits a true Tm relationship. More recently, it has been observed, in man, that an increased renal excretion of phosphate follows the administration of cortisone and ACTH. Moreover, negative phosphate balance, depletion of intracellular stores of phosphate and demineralization of bone have been observed in conditions of hyper-adrenocorticism (Albright 1942, Eliel *et al.*, 1951; Ingbar *et al.*, 1951; Pearson *et al.*, 1950; Freeman *et al.*, 1950; Soffer *et al.*, 1950). Although logically inferred, it has not been clearly defined that this increment in phosphate excretion results from an alteration in the renal tubular capacity for phosphate reabsorption consequent upon an excess of adrenal hormone. Accordingly the series of experiments described below was performed to determine the effects of cortisone and desoxycorticosterone on the renal tubular reabsorption of phosphate. Since exchange of hydrogen and potassium ions for sodium bound by urinary buffers plays a significant role in sodium conservation (Berliner, 1951, Pitts 1950), the effects of these steroids on titratable acid and potassium excretion were observed simultaneously. These experiments show that cortisone depresses the renal tubular reabsorptive capacity for phosphate, whereas desoxycorticosterone is without effect. On the other hand, no clear cut effects of either steroid could be demonstrated on the renal mechanisms involved in the excretion of titratable acid and potassium.

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[2] This work was done during the tenure of a research fellowship of The American Heart Association.

METHODS

In all, 21 experiments were performed on 4 normal and 2 adrenalectomized dogs, both untreated and treated with cortisone or desoxycorticosterone.[3] Prior to the experiments on adrenalectomized dogs, hormone treatment was discontinued for 5–7 days and the animals were maintained with 0.6% salt solution *ad lib.* during this interval. In order to define the maximal tubular capacity for reabsorption of phosphate, the plasma phosphate level and thus the amount presented to the renal tubules were increased by the infusion of neutral sodium phosphate. In some of the experiments the plasma phosphate level was elevated in stepwise fashion; in others a constant infusion of phosphate was maintained. Cortisone, in amounts of 50 to 75 mgm., or desoxycorticosterone, 2 to 5 mgm., was administered intravenously either prior to, or during the course of the experiment. Cortisone acetate was given as a microcrystalline suspension in saline. Dexosycorticosterone was given as the crystalline preparation dissolved in sesame oil and homogenized with saline. Creatinine, which was used to measure the rate of glomerular filtration, was infused with the phosphate in amounts sufficient to maintain optimum plasma levels. Sodium and potassium concentrations of plasma and urine were determined with an internal standard flame photometer. Creatinine was determined colorimetrically by the method of Bonsnes (Bonsnes, 1945). Titratable acid excretion was determined by electrometric titration as described in a previous communication (Pitts *et al.*, 1945). Phosphate was determined on trichloracetic acid filtrates of plasma and diluted urine by the method of Fiske and Subbarow (1925) as modified by Pitts (1933).

RESULTS

Table 1 summarizes the data of three typical experiments performed on three dogs and demonstrates the effect of the administration of cortisone or desoxycorticosterone on the reabsorption of phosphate. In these experiments constant infusions of neutral phosphate were instituted 30 min. prior to the first control period and maintained throughout the experiment. Following two control periods of twenty minutes, the steroid was given and the effect upon phosphate reabsorption, potassium and titratable acidity measured over a period of 60–80 minutes. The experiment at the top of the table illustrates the decrease in tubular reabsorption of phosphate which follows the injection of 50 mg. of cortisone in a normal dog. This occurs within a thirty-minute period following steroid administration. The second experiment shows that desoxycorticosterone has no effect on the renal tubular reabsorption of phosphate. The third experiment demonstrates that cortisone caused a decrease in phosphate reabsorption in an adrenalectomized dog maintained without hormone substitution therapy for six days which was similar to that seen in the normal animal.

Figure 1 shows graphically the average change in phosphate Tm resulting from adrenal steroid injection in four normal dogs. Each box represents the average phosphate reabsorption in mM./min. over a period of 90 to

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[3] We are greatly indebted to the Schering Corporation for the generous supply of desoxycorticosterone used in these experiments.

100 minutes in untreated dogs and following the administration of cortisone or desoxycorticosterone. The average phosphate Tm was calculated by averaging the phosphate reabsorbed in 3–5 different experiments on each dog. From this, it can be seen that cortisone significantly reduced phosphate reabsorption whereas desoxycorticosterone had no such effect.

TABLE 1. THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE PHOSPHATE REABSORPTION IN TWO NORMAL DOGS (1 AND 2) AND ONE ADRENALECTOMIZED DOG (3)

Urine flow cc./min.	Glomerular filtration rate,	Plasma		Phosphate			Excreted	
		Phosphate mM/L	Potassium mM/L	Filtered	Excreted	Reabsorbed	Titractable acid	Potassium
				mM/min.			mM/min.	
				1 Control				
1.9	75.7	4.26	3.64	.322	.211	.111	.046	.086
2.31	75.5	5.29	3.14	.405	.305	.100	.056	.094
				Cortisone				
3.7	74.8	5.9	3.14	.441	.379	.062	.056	.073
4.8	75.5	6.23	3.0	.470	.430	.040	.075	.089
6.2	81.2	6.48	2.7	.526	.476	.050	.080	.131
				2 Control				
1.2	103	4.07	3.85	.419	.262	.157	.052	.092
1.4	94	4.29	3.85	.403	.266	.137	.050	.072
				Desoxycorticosterone				
3.3	94	4.65	3.85	.435	.304	.131	.107	.063
4.1	102	4.77	3.8	.487	.343	.144	.095	.108
3.8	106	4.80	2.6	.509	.359	.150	.085	.139
				3 Adrenalectomized Control				
2.6	68	5.96	5.5	.405	.203	.202	.159	.063
3.4	69	6.07	5.8	.418	.224	.194	.152	.082
				Cortisone				
2.9	61	5.84	5.5	.356	.228	.128	.110	.128
3.0	61	5.42	5.0	.330	.217	.113	.097	.135
2.7	66	4.95	4.5	.327	.204	.123	.095	.142

TITRATABLE ACID AND POTASSIUM EXCRETION

Figure 2 shows the increase in potassium and titratable acid excretion which occurs as increasing amounts of phosphate are filtered and thus excreted, under normal circumstances and under the influence of cortisone. In the upper part of the figure the sum of potassium and titratable acid, as a measure of sodium conservation, is plotted as a solid line. In the lower part of the figure is shown the increment in sodium excretion which occurs simultaneously with the increased excretion of titratable acid and potassium. In this pair of experiments the amounts of these substances excreted are seen to be somewhat increased following cortisone, especially when smaller amounts of phosphate were filtered. We have presumed this to be related to the decrease in phosphate Tm. The resulting increase in phosphate excretion per unit filtered led to an increase in sodium, potassium and hydrogen ion excretion. However this has not been a consistent finding. In all animals, the general pattern of titratable acid and potassium excretion was not significantly altered by the administration of adrenal steroid. Further, the response of the adrenalectomized salt-maintained animal did not differ from the normal when the animal was presented with a phosphate load. Reference to Table 1 shows that the adrenalectomized animal excretes considerable amounts of titratable acid and potassium following administration of phosphate. Similar results were obtained in 6 experiments on 2 adrenalectomized dogs.

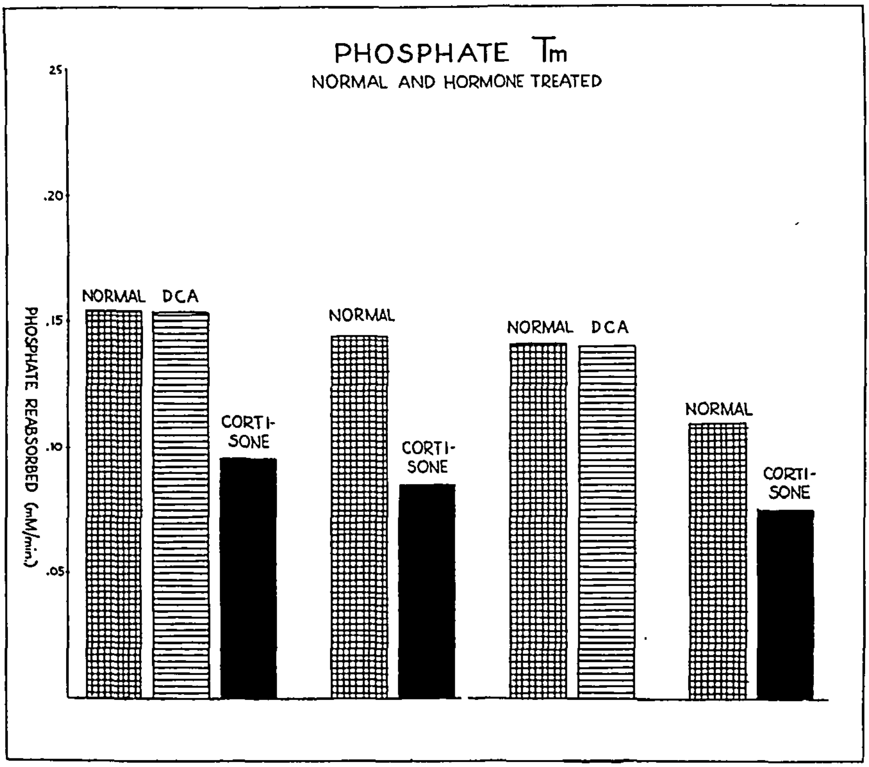

FIG. 1. Effects of cortisone and desoxycorticosterone on the average phosphate reabsorption in four normal dogs.

Figure 3 illustrates further that no specific quantitative differences in titratable acid excretion could be attributed to the influence of adrenal steroid. In this figure is shown the rise in titratable acid excretion which occurs as increased amounts of phosphate are excreted in the normal and adrenalectomized dog, both with and without adrenal hormone. No consistent differences were noted between normal animals and those subjected to adrenalectomy, nor was the excretion of hydrogen ions effected by cortisone or desoxycorticosterone.

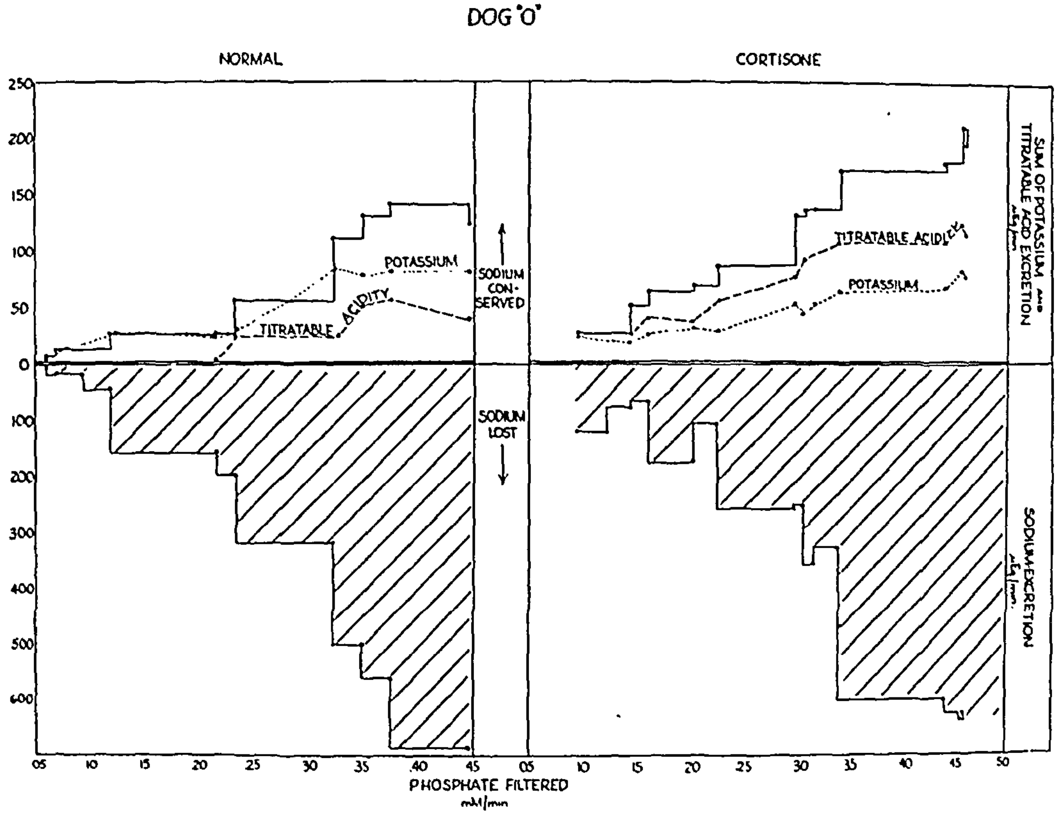

FIG. 2. Sodium, potassium and titratable acid excretion during phosphate loading in a dog with and without cortisone.

DISCUSSION

From the data presented above, it is evident that the depression of phosphate Tm following cortisone administration may be a factor in bringing about the negative phosphate balance observed following the administration of cortisone or ACTH. The increase in the renal excretion of phosphate if continued sufficiently long could account for intracellular depletion of phosphate.

Although other factors may be operative in bringing about the alterations in phosphate metabolism observed in hyperadrenocorticism, these experiments do not elucidate the nature of these mechanisms; nor was any attempt made in these experiments to compare the relative significance of a reduction in phosphate Tm to other factors known to influence phosphate metabolism.

Since the adrenalectomized rat and the Addisonian patient have been reported to show defects in ammonium and titratable acid excretion, it might be expected that the adrenalectomized dog would display a renal deficit in the exchange of hydrogen and potassium ions for sodium (Loeb *et al.*, 1933; Pitts, 1950; Stahl *et al.*, 1936). Such was not found to be the case in our experiments. When presented with a phosphate load, the excretion of titratable acid and potassium did not differ significantly from that seen in normal animals. Further, this response was not modified by cortisone. In fact, no clear cut effects of either cortisone or desoxycorticosterone could be demonstrated upon the excretion of these substances per se: any alterations which did occur appeared to be secondary to those alterations in phosphate excretion which followed cortisone administration. From this, we infer that cortisone does not exert a major influence directly on the renal tubular mechanisms involved with the exchange of hydrogen and potassium for sodium in the dog.

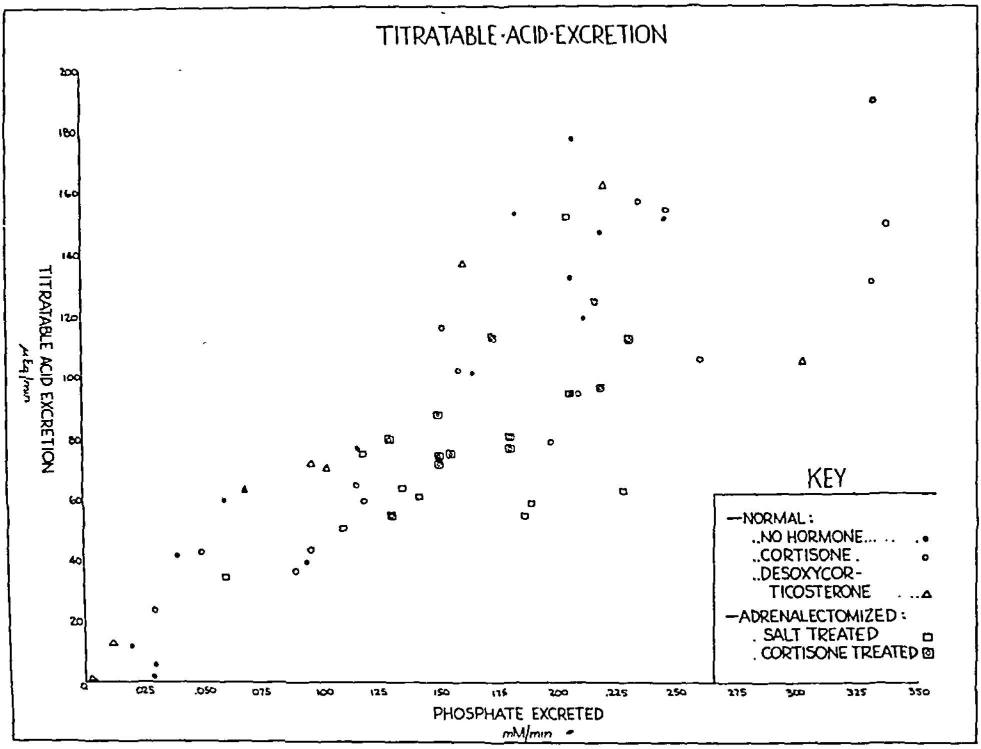

FIG. 3. Titratable acid excretion plotted as a function of phosphate excretion in normal, adrenalectomized and hormone-treated dogs.

CONCLUSIONS

(1) The renal tubular reabsorptive capacity for phosphate is decreased following the administration of cortisone in intact and adrenalectomized dogs. (2) Desoxycorticosterone does not depress the phosphate Tm. (3) The increase in excretion of titratable acid and potassium, which occurs as increasing amounts of phosphate are excreted, is not significantly altered by administration of cortisone or desoxycorticosterone. (4) Adrenalectomized animals maintained on sodium chloride show the same increase in the excretion of titratable acid and potassium following phosphate loading, as that seen in intact animals given a similar phosphate load.

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CYTO-PHYSIOLOGIC ASPECTS OF THYROTROPHIC HORMONE SECRETION IN THE GOITROUS GUINEA PIG*

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THE assignment of cell types to specific hormone secretions in the adenohypophysis continues to thwart proper understanding of the mechanisms underlying thyroid-hypophyseal interplay. Earlier opinion (Severinghaus, 1937) favored the pituitary acidophile as the source of thyroid stimulating hormone (TSH) but recent investigations, based largely on the rat, more clearly implicate the basophiles (Griesbach and Purves, 1945; Brolin, 1945; Goldberg and Chaikoff, 1949; and Halmi, 1950). Purves and Griesbach (1951) moreover, have applied histochemical procedures to the rat adenohypophysis which allegedly discriminate between thyrotrophic and gonadotrophic hormone producing cells routinely classed as basophiles. Despite the apparent relationship between thyroid activation and pituitary basophilia (D'Angelo, 1941; Griesbach, 1951), cellular localization of TSH production in the hypophysis is not satisfactorily established. The general failure to demonstrate increased content of TSH in the rat pituitary after procedures (thyroxine deficiency, cold) which presumably activate the thyrotrophic mechanism clouds interpretation of its morphology (Kuschinsky, 1935; Turner and Cupps, 1940; Griesbach and Purves, 1943). The need for histophysiologic studies of thyroid-pituitary interaction in species other than the rat is consequently urgent. The relatively low content of TSH in the blood and hypophysis of the guinea pig (D'Angelo and Gordon, 1950) suggests a type of thyroid-pituitary balance which may respond markedly to proper endocrine manipulation. Accordingly, a detailed investigation of thyroid and pituitary parameters is being made in this form, one phase of which has already been reported (D'Angelo, Paschkis, Cantarow, Siegel, and Rivero-Fontan, 1951). In the present study, blood and pituitary TSH levels have been determined and correlated with morphologic changes in the thyroid and hypophysis of guinea pigs made hypothyroid with an anti-thyroid agent.

METHOD

A total of 110 normal and experimental guinea pigs were used. Animals received propylthiouracil (0.1%) in the basic ration (Purina Omolene) for periods of 7, 40, and 80

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days, with control animals receiving the basic ration alone. At sacrifice, the thyroid and hypophysis were quickly removed, weighed on a microtorsion balance, and prepared for microscopic study. Pituitaries were divided sagittally, with portions fixed either in Helly's of Champy's fluid. Tissue from the latter were post-osmicated (4–5 days at 37° C.) to demonstrate the Golgi apparatus. Serial sections of paraffin-embedded pituitaries were cut at 2, 3 and 4 micra, and were stained with modified Masson or azan procedures. Thyroid response was evaluated by microhistometric measurement of the acinar cell height (a total of 200 follicular cells per thyroid lobe was measured). Blood for the bioassay study was obtained by cardiac puncture under light ether anesthesia. The sera of animals in respective control and experimental groups were pooled and frozen until ready for assay by the stasis tadpole technic (D'Angelo and Gordon, 1950). The pituitary extract was prepared by a standardized procedure used in the laboratory. Seven anterior lobes (posterior lobe removed by blunt dissection) were homogenized in acidified saline (0.25% glacial acetic acid). The material was extracted twice and, after centrifugation, the clear supernatant fluid was bioassayed for TSH in the tadpoles.[1] Extract volumes were adjusted so that each test animal received an equivalent of 1.4 milligrams of anterior pituitary tissue over a period of 4 injections. For blood assay a total of 0.30 cc. of serum was given in 6 injections.

TABLE 1. BLOOD THYROID-TSH BALANCE AND TSH CONTENT OF THE ANTERIOR PITUITARY IN THE GOITROUS GUINEA PIG

Mean body wgt.-gm. (no. pigs)	Days on drug	M. thyroid wgt.-mg.	M. thyroid cell hgt. (μ)	M. ant. pit.-mg.	Ant. pit. assay		Blood assay	
		100 gm. body wgt.		100 gm. body wgt.	Thy. cell hgt. (μ)	Est. TSH in extract	H-limb increase (mm.)	thyroid cell hgt. (μ)
558±75[1] (7)	0	15.1±1.6	7.1±0.2	1.6±0.07	7.8±0.3	1.6μg.[2] (0.8–2.4)	2.4±0.1[3]	5.1±0.1[3]
435±50 (7)	7	17.2±0.9	7.5±0.3	1.8±0.15	6.9±0.2	0.4μg. (0.2–0.3)	2.4±0.1	5.9±0.1
384±20 (7)	40	26.8±2.6	12.0±0.9	1.9±0.12	8.6±0.3	4.0μg. (2.5–6.0)	1.8±0.1	5.8±0.1
486±50 (7)	78	68.9±9.5	12.2±0.3	2.2±0.09	9.5±0.2	14.0μg. (10–20)	1.2±0.1	5.9±0.2
357±30 (7)	0	16.1±1.0	7.1±0.3	1.8±0.08	7.6±0.2	1.2μg. (0.5–1.6)	2.6±0.2	5.3±0.1

[1] Mean body wgt. at sacrifice ± avg. dev.; in all other columns ± values represent standard error of mean.

[2] TSH content of guinea pig pituitary extracts given in mean microgram equivalents (range) of a previously standardized thyrotropin preparation (D'Angelo, Endocrinology 48: 249, 1951).

[3] Refers to mean hind-limb increase and thyroid cell height in stasis tadpoles receiving guinea pig sera.

RESULTS

The summarized data (Table 1) confirm the earlier finding (D'Angelo *et al.*, 1951) that there is appreciable delay in the onset of morphologic change in the thyroid. Neither thyroid weight nor acinar cell height was changed from normal with seven days treatment of propylthiouracil. The TSH content of pituitary and serum was significantly altered, however. TSH concentration in the adenohypophysis decreased to one-fourth of normal, whereas, blood TSH, not detectable in normal guinea pig serum, rose to levels approximating 0.001 JSU[2]/ml. With continued treatment, elevation of the thyroid cell height, colloid release, and hyperplasia occurred (Fig. 1). The anterior pituitary eventually enlarged, and TSH concentrations in the gland were markedly increased (approximately 280% and 1000% of normal at 40 and 78 days respectively). The elevated blood TSH titers were maintained at a relatively constant level throughout. The decreased hindlimb extension in tadpoles given such sera indicated that the heightened TSH titers were accompanied by simultaneously lowered levels of thyroid hormone. This shift in the thyroid-TSH blood equilibrium follows the inhibition of thyroid hormone formation with goitrogen (D'Angelo and Gordon, 1950).

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[1] Grateful acknowledgment is made to Mrs. Ada Schafer and Mrs. Betsey Brownlee for technical assistance.

The thyrotrophic hormone shifts in blood and hypophysis, as well as the morphologic changes in the thyroid, appeared to be closely dependent upon alterations in the pituitary basophiles. (The detailed changes in hypophysial cell types, as related to disturbances in the thyroid-pituitary axis, will be considered elsewhere; only the major changes which occur with goitrogen will be given here.) The basophilic population, normally concentrated around the area of the stalk along the median, ventral, aspect (bone surface) of the gland showed definite structural changes as early as several days following propylthiouracil treatment. These involved a decrease in the numbers of large, finely granular, deeply staining basophiles and a simultaneous increase in small, coarsely granular cells with hyperchromatic nuclei (Figs. 2 and 3). After prolonged treatment—when TSH concentrations in the gland were appreciably augmented—there was marked hypertrophy and hyperplasia of both light and deeply staining varieties of basophiles, their distribution in the gland becoming widespread (Fig. 4). The dark, irregularly-shaped basophiles were unusually large. These cells were further characterized by a fine cytoplasmic granulation, hypertrophied Golgi networks, and by large nuclei bearing numerous prominent nucleoli (Figs. 5 and 6). Vacuolation was rare, however, and no obvious counterpart to the so-called "thyroidectomy" or "goiter" cell of the rat appeared. There did appear after prolonged treatment, however, certain rather large basophilic cells in the stalk area displaying a coarsely punctated granulation in an otherwise clear chromophobic cytoplasm. Although the acidophiles remained numerous throughout treatment, an appreciable proportion of these cells showed regressive changes. Cytoplasm was visible only as rims or crescents of sparsely granular acidophilic material. Nuclei were pycnotic and the Golgi appratus became inconspicuous. Many acidophiles retained the normal structural pattern, however.

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[2] Junkmann-Schoeller Units.

DISCUSSION

The initial diminution and subsequent marked rise in pituitary TSH content found with chronic goitrogen treatment indicates the necessity of considering the duration factor in cyto-physiologic studies of the adenohypophysis. Failure to consider this may well account for the sharply conflicting reports regarding TSH content of the rat pituitary in thyroxine deficiency (Griesbach and Purves, 1943; Turner and Cupps, 1940; Gordon *et al.*, 1945). The importance of accurate definition of blood TSH levels in conjunction with pituitary examination is equally obvious. It can be safely inferred from the present study that the inhibition of thyroid hormone formation with propylthiouracil elicits augmented release of TSH from the pituitary. With short periods of treatment, thyrotrophic hormone release into the circulation must occur before the mechanism of production is appreciably activated. As thyroid hormone deficiency continues, TSH production eventually surpasses release, so that hormone stores in the pituitary now augment. The relatively constant blood levels of the hormone throughout may mean that delivery of TSH into the circulation, under these conditions of thyroid hormone deficiency, is at a maximal rate. The possibility still exists, however, that increased production of TSH in the pituitary is accompanied by an increased delivery rate of the hormone into the blood with this augmented release of TSH counterbalanced, in turn, by excessive destruction or inactivation in the circulation.

The strong basophilia of the pituitary found at a time when TSH content of blood and hypophysis is high makes it more than probable that the genesis of TSH resides in this cell type. The persistence of the acidophiles in the hypophysis of the goitrous guinea pig, as contrasted with their disappearance in the rat, does not complicate interpretation of the cytologic picture. Neither in numbers, granule content, nor in the behavior of the cytoplasmic organoids, can the acidophiles be correlated with change in TSH stores of the pituitary. The association of this cell type with continued

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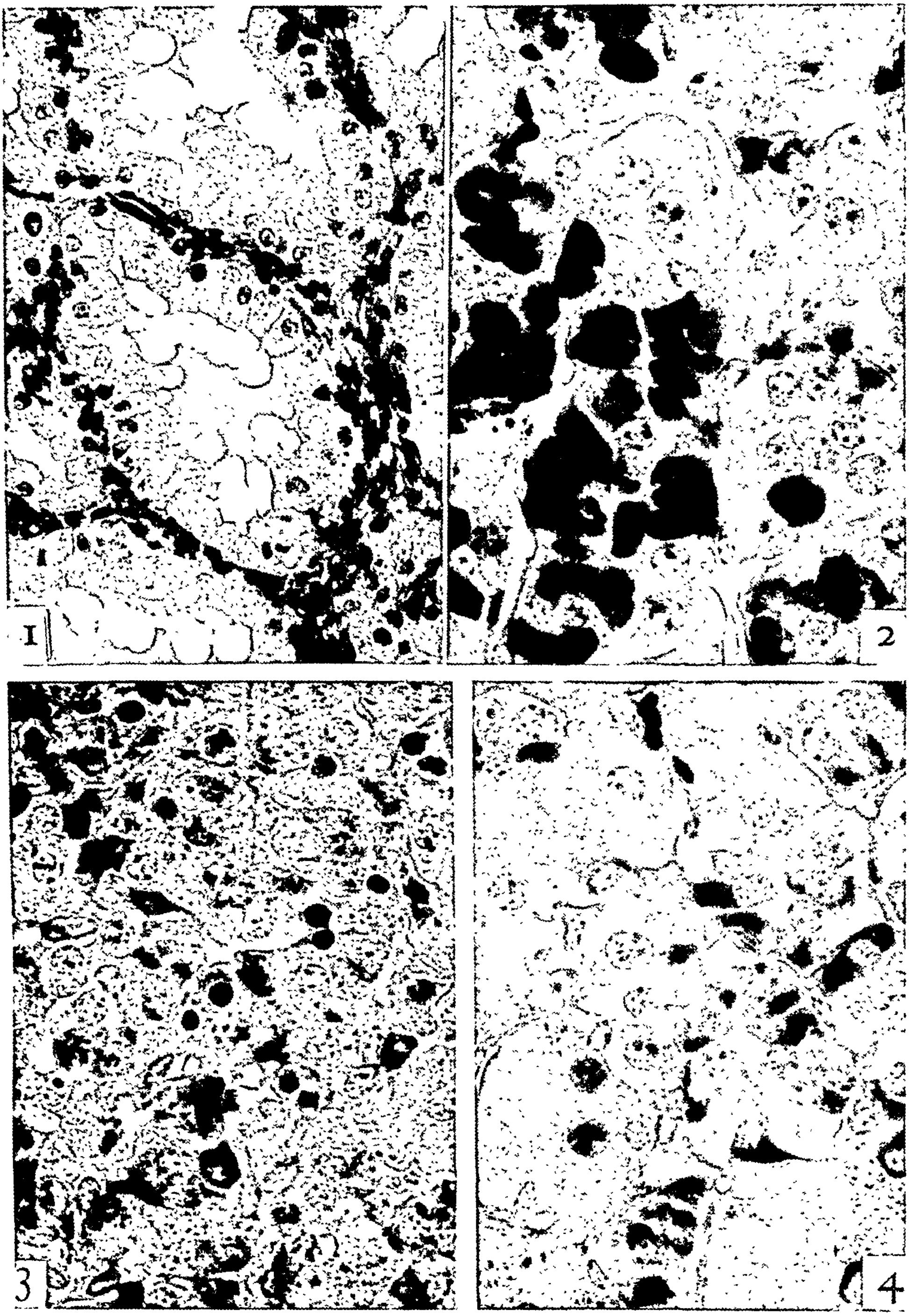
EXPLANATION OF FIGS. 1–4

FIG. 1. The histologic response of the thyroid gland after 78 days treatment with goitrogen (Bouin's Picro-Formol; H. and E., ×350).

FIG. 2. A section through the normal adenohypophysis displaying several large basophiles (upper right) and the more numerous smaller acidophiles (black, elongate elements at left; Helly's, Masson, ×700).

FIG. 3. The widespread degranulation and diminution in size of the chromophiles in the pituitary after 5 days on drug (Helly's, Masson, ×700).

FIG. 4. The adenohypophysis of a guinea pig after 85 days on goitrogen. The basophiles (grey) are hyperplastic; acidophiles (black) are small with rims or crescents of granular cytoplasm (Helly's, Masson, ×700).



Figs. 1–4

EFFECT OF HYPOPHYSECTOMY ON I^{131} METABOLISM IN NORMAL AND THIOURACIL TREATED C_3H MICE

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INTRODUCTION

MANY studies have been made of the effect of hypophysectomy on the radioiodine metabolism of the rat (Leblond, Sue and Chamorro, 1940; Leblond and Sue, 1941; Morton, Perlman, Anderson and Chaikoff, 1942; Cortell and Rawson, 1944; Chaikoff and Taurog, 1949; VanderLaan and Greer, 1950; Ghcsh, Woodbury and Sayers, 1951; Randall and Albert, 1951; Randall, Lorenz and Albert, 1951; Albert, Tenney and Lorenz, 1952). Similar studies in the mouse have not been reported as yet.

This paper presents the experimental findings made in a study of some effects of hypophysectomy on I^{131} metabolism in C_3H mice with either normal or hyperplastic thyroid glands.[1] Data are reported on the I^{131} uptake by the thyroid gland, on the percent of thyroid I^{131} in thyroxine, and on the concentration of radioiodide in the thyroid gland compared to that in the serum. It was found that the I^{131} content of the thyroid gland 25 hours after I^{131} injection undergoes an extensive and progressive decrease with increasing time intervals after hypophysectomy. In contrast, while the ratio of radiodide content of the thyroid gland to that of serum decreases after hypophysectomy, it remains elevated above 50.

METHODS AND MATERIALS

The radioiodine studies were made in male C_3H mice between four and five months of age. The mice were supplied by the Animal Production Unit of the National Institutes of Health.

Diet: Two groups of mice were studied. One group was fed from weaning a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner, Morris, 1952). After weaning, the other group was fed for three months a meal diet similar in composition to the stock diet but containing a supplement of 2.5 gm. of thiouracil per kilogram. It was then fed the stock diet without supplement for the remainder of the experiment.

Operative procedures: Hypophysectomy was performed through a parapharyngeal approach (Korteweg and Thomas, 1939) when the mice were about four months old. In the sham operation, the hypophysectomy procedure was followed up to and including

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[1] A preliminary report of these results was presented at the New York meeting of the Federated Societies in April 1952. (Wollman and Scow, 1952.)

the removal of the outer table of the occipital bone. Only those hypophysectomized mice are included in this report in which complete extirpation of the gland had been achieved as ascertained by visual examination (9×magnification) of the sella turcica and adjacent tissues. Serial sections of the sella turcica and adjacent bone, stained with hemotoxylin and eosin, were made of the hypophysectomized mice in certain groups as evidence of completeness of the operation. Testes weights were obtained in certain groups of normal and hypophysectomized mice.

I^{131} measurements: The I^{131} in thyroid glands or in extracts of thyroid glands was measured by gamma counting (Wollman, Morris and Green, 1951). The I^{131} in serum was measured by adding 0.05 ml. serum to 0.95 ml. of H_2O containing a mg. of carrier NaI. An aliquot of this solution was dried on a copper planchet and counted with an end window counter. The statistical counting error was less than three per cent by either method. The counting rate of a sample using the beta counter was 150 times that using the gamma counter.

Uptake 25 hours after I^{131} injection: In the 25-hour uptake studies, each mouse received intraperitoneally 10 μc. of carrier free I^{131}[2] and was killed with ether 25 hours later. The thyroid gland, after weighing, was placed in 1 ml. 2 N NaOH and its I^{131} content was determined. The thyroid gland of each mouse fed the thiouracil-free diet was then hydrolyzed on a steam bath, and the thyroxine fraction was isolated by the butanol extraction method of Taurog and Chaikoff (1946) and its I^{131} content determined.

Uptake at 1½ hours after I^{131} injection: One and one-half hours after receiving 10 μc. of I^{131} as described earlier, each mouse was anaesthetized with ether and bled from the heart. The thyroid gland was removed immediately thereafter and was placed in a glass homogenizer with 3 ml. of ice cold 8 per cent trichloroacetic acid solution containing a few mg. of NaI as carrier. It was maintained at a temperature below 5° C. for the remainder of the procedure. The I^{131} content was determined and the tissue was then homogenized in 8 ml. of the acid, centrifuged. and the I^{131} content of the supernatant was measured. The serum I^{131} was determined. Test showed that a negligible fraction of the serum I^{131} was protein bound at this time.

The thyroid iodide/serum iodide concentration ratio: Each mouse in this series was given a subcutaneous injection of 0.5 ml. of freshly prepared solution containing 5 mg. propylthiouracil per ml. of 0.9 per cent NaCl which contained NaOH at a concentration of 0.04 normal. One hour later 10 μc. I^{131} was given intraperitoneally. One to two hours after I^{131} injection the mouse was anaesthetized with ether, a blood sample taken from the heart, and the thyroid gland was immediately removed and weighed. The I^{131} content of thyroid gland and serum were measured.

RESULTS

There was a body weight loss which varied from 4 to 6 grams in hypophysectomized mice (mean body weight at surgery was 30 gm.) during the first 4 to 7 postoperative days after which the weight remained unchanged. The three gram weight loss in the shams was transient; their body weights were back to preoperative values in 7 days. In the mice fed the stock diet, the thyroid weight, which was 2 mg. in normals, was decreased by 26 per cent at 7 days after hypophysectomy and by 39 per cent at 12 days. In mice fed the thiouracil diet the thyroid glands averaged 19 mg. The thyroid weights of mice fed the thiouracil diet decreased by 15 per cent during the 13 days following hypophysectomy. However, the thyroid weights of the sham-operated controls decreased sufficiently so that the thyroid weights in the experimental and control groups never differed significantly. The mean and the standard deviation of the weights of a single testis in mice fed the thiouracil supplement was 86 ± 7 mg. in the shams, whereas in hypophysectomized mice it was 77 ± 7 mg. at six days after hypophysectomy and 40 ± 11 mg. at 13 days.

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[2] The I^{131} used in this investigation was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

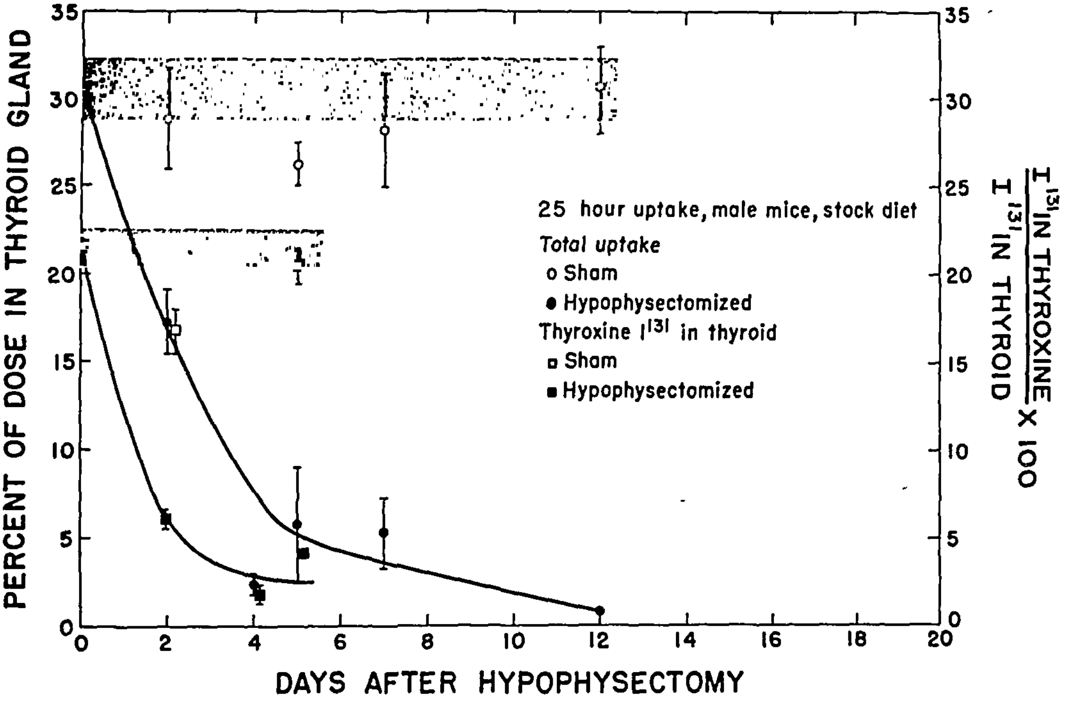

FIG. 1. Effect of hypophysectomy on I^{131} uptake and on percentage of thyroid I^{131} present in thyroxine fraction 25 hours after radioiodide administration in C_3H mice fed the stock diet. The shaded areas indicate the standard error range for the normals; the upper is for I^{131} uptake, the lower for thyroxine fraction. The vertical bar at each point represents the standard error. Four to six mice were used per point. The point for five days after hypophysectomy includes observations on only two mice.

The 25-hour I^{131} uptake at various time intervals after hypophysectomy in mice fed the stock diet is shown in Figure 1. There was a progressive decrease in the uptake of the thyroid glands in hypophysectomized mice during the postoperative period, with the uptake decreasing to 50 per cent of the control value by the second postoperative day.[3] The sham-operative procedure had no significant effect on the uptake even though the left lobe of the thyroid gland had been compressed during the operative procedure.

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[3] In a few data collected on female mice the I^{131} uptake was 30 per cent higher than in corresponding male mice, but there was no significant sex difference in the I^{131} uptake in mice that had been hypophysectomized seven days.

The per cent of thyroid I^{131} in the thyroxine fraction in these mice is also presented in Figure 1. By the second postoperative day, a marked reduction of the per cent of thyroid I^{131} in the thyroxine fraction to one-third of that for the shams was observed in hypophysectomized mice, there being a further decrease thereafter.

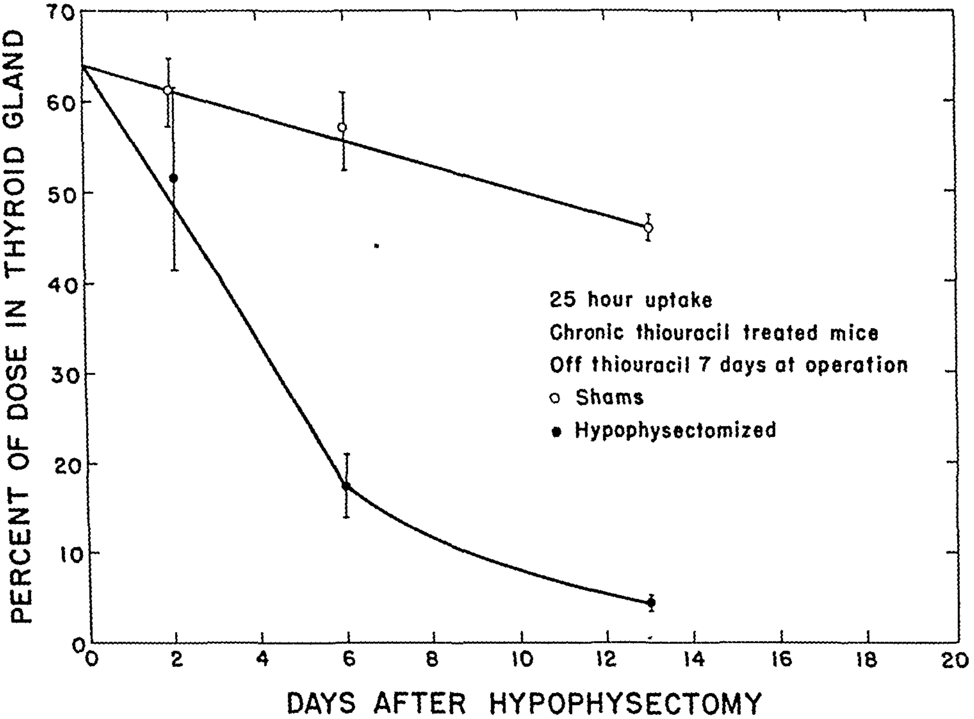

FIG. 2. Effect of hypophysectomy on uptake of radioiodide 25 hours after administration in C_3H mice fed 0.25% thiouracil diet up to seventh preoperative day and then fed the stock diet. Approximately 5 hypophysectomized mice and 6 sham operated mice were studied at each time interval.

The 25-hour I^{131} uptake in hypophysectomized and sham-operated mice fed the thiouracil diet is presented in Figure 2. The mice were fed the stock diet for seven days just prior to operation. Completeness of hypophysectomy was verified by examination of serial sections of the region of the sella turcica in those mice studied at 13 days after hypophysectomy. There was a progressive decrease in the I^{131} uptake following hypophysectomy in these animals, as observed also in those fed the stock diet. The gradual decrease in I^{131} uptake with increase in the postoperative time intervals observed in sham-operated mice probably was the result of the withdrawal of thiouracil. The amount of I^{131} incorporated into the thyroxine fraction in these hypertrophied thyroids was not determined since preliminary studies had shown that it was too small to be measured accurately.

The effect of hypophysectomy on I^{131} uptake 1.5 hours after I^{131} injection in mice fed the thiouracil diet is shown in Table 1. The mice were fed stock diet for 18 days before operation. Completeness of hypophysectomy was verified in each experimental animal by examination of serial sections of the region of the sella turcica. This study was made to check the possibility that the small effect of hypophysectomy on 25-hour uptake at short time intervals after operation in animals fed thiouracil might be due in part to the fact that the maximum uptake in the thyroid glands of the controls occurs only a few hours after I^{131} injection, while the uptake in the hypophysectomized animals increases more slowly, but continues to increase for a much longer time after injection. At this 1.5 hour time interval the total I^{131} in the thyroid glands of the hypophysectomized mice was only 20 per cent that of the controls, the difference being almost twice as great as that observed at 25 hours after I^{131} injection. The concentration of acid-soluble I^{131} in thyroid glands and the concentration of serum I^{131} are approximately 50% higher in the hypophysectomized mice than in the controls. As a consequence, the ratio of the concentration of acid-soluble I^{131} in the thyroid to serum radioiodide concentration was the same for both

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON I^{131} IN THE THYROID GLAND AND SERUM IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS

		Hypophysectomized	Sham-operated
Number of mice		5	5
Thyroid I^{131}			
Total	counts/sec./100 mg.[1]	109 ±6	495 ±29
TCA-soluble	counts/sec./100 mg.	66.5 ±6	41.2 ± 5
Per cent TCA-soluble		61 ±4	8.3 ± 0.3
Per cent of injected dose			
Total		2.92 ±0.36	16.6 ± 1.5
TCA-precipitable		1.11 ±0.12	15.3 ± 1.5
Serum I^{131}	counts/sec./100 mg.[1]	4.11 ±0.26	2.62 ± 0.06
$\frac{\text{Thyroid iodide conc.}}{\text{Serum iodide conc.}}$		14.5 ±1.2	16.2 ± 1.9

[1] All counting rates reported as if measured by gamma counting. The serum data were obtained by beta counting, and were converted to the equivalent gamma counting data using an experimentally determined conversion factor.

Mice fed diet containing thiouracil for three months, fed stock diet 18 days, and then operated on. Studies were made on sixth postoperative day. Samples were taken one and one-half hours after I^{131} administration.

groups. Acid-soluble I^{131} amounted to 60% of the total I^{131} in the thyroids of the hypophysectomized mice, but only 8% in the controls.[4] The amount of organic bound I^{131} in the thyroids of hypophysectomized mice was much less than in those of the controls.

The effect of hypophysectomy on the thyroid iodide/serum iodide concentration ratio in mice fed thiouracil is presented in Table 2. A statistically significant though small depression of this ratio as the result of hypophysectomy was observed only at thirteen days after operation, the latest time interval studied. The hypophysectomies in this last group, studied 13 days after operation, were shown to be complete by examination of serial sections of the region of the sella turcica. Two and one-half mg. of propylthiouracil was shown to be effective in blocking protein binding of radioiodide by the thyroid gland for at least five hours in these mice. The values of the ratios in the sham-operated mice were abnormally low, since mice fed either a goitrogen-free diet or thiouracil in this laboratory ordinarily have ratios of over 200. The low values observed may have been due to a somewhat higher iodide concentration in the particular batch of stock diet fed the mice in the time interval during which these studies were made.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON THYROID IODINE/SERUM IODIDE CONCENTRATION RATIO[1] IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS[2]

Days after operation	Concentration ratio	
	Hypophysectomized	Sham-operated
2	75 ± 8 (5)[3]	96 ± 4 (7)
6	90 ± 10 (6)	91 ± 9 (7)
13	67 ± 6 (6)	108 ± 9 (6)

[1] In order to prevent organic binding of radioiodide, each mouse was given a single subcutaneous injection of a solution containing 2.5 mg. of propylthiouracil one hour prior to injection of 10 μc of I^{131}. Samples were taken one to two hours after the I^{131} injection.

[2] Fed thiouracil diet three months, hypophysectomized after feeding stock diet seven days.

[3] Mean ± standard error; parenthesis encloses the number of animals examined.

DISCUSSION

The effects of hypophysectomy on the I^{131} metabolism in the mouse appear qualitatively similar to those in the rat. The results, however, suggest some interpretations of the effects of hypophysectomy which are different from interpretations in papers on the rat.

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration decreased progressively with increasing time intervals after hypophysectomy in mice fed either the stock diet or the thiouracil-containing diet. Although not shown in figure 1 the logarithm of the uptake of the thyroid glands decreased linearly with the time after hypophysectomy in the mice fed the stock diet. This indicates a constant fractional rate of decrease of the 25-hour uptake with time after hypophysectomy. This interpretation may be contrasted with that suggested by Randall and Albert (1951) who concluded that the thyroid activity in the rat reached a minimum of 10% of normal by six days after hypophysectomy (using maximum uptake of injected I^{131} as a measure of thyroid activity). The interpretation of these data on the rat as well as the present 25-hour uptake data is complicated by the fact that the maximum uptake reflects not only thyroid function but also kidney function, and in particular the fact that radioiodide excretion by the kidney is grossly decreased in the hypophysectomized animal (Albert, Tenney and Lorenz, 1952). This would tend to make injected I^{131} available to the thyroid gland in the hypophysectomized animal for a longer time than in the normal, and tend to decrease the apparent loss of thyroid activity if a long time interval between injection and thyroid I^{131} measurement is selected. For a reliable measure of thyroid activity it is important to study a characteristic which is relatively independent of kidney activity such as thyroid clearance or uptake at times so soon after I^{131} administration that only a small fraction of administered I^{131} could be excreted by the kidney even in the normal animals.

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[4] Albert and Lorenz (1951) have obtained similar results in the rat.

It is important to ascertain which of the factors involved in the accumulation of I^{131} by the thyroid gland are grossly affected after hypophysectomy and responsible for the observed decreased accumulation. The preliminary observations on organic bound I^{131} six days after hypophysectomy in the mice with hyperplastic thyroid glands indicates a marked decrease in organic bound I^{131} in the hypophysectomized mice. This correlates well with the prompt decrease in the proportion of thyroid I^{131} found in the thyroxine fraction in mice fed the stock diet and previously reported for the rat (Morton, Perlman, Anderson and Chaikoff, 1942). Both organic binding of I^{131} and formation of thyroxine are presumably linked to oxidative processes in the cell.

The decrease in the organic bound I^{131} in the thyroid glands of the hypophysectomized mice with hyperplastic glands does not appear to be due to a lack of radioiodide in the gland since the acid-soluble I^{131} was actually higher in their glands than in those of the control mice. If the relationships at 1.5 hours after I^{131} injection are representative of those at earlier times it would suggest that the process of organic binding of I^{131} was grossly impaired in hypophysectomized mice.

The values reported for thyroid iodide/serum iodide concentration ratios of mice hypophysectomized after chronic feeding of thiouracil are almost always above 50. Many additional measurements of this ratio in mice hypophysectomized after feeding a thiouracil-free diet since weaning, to be reported elsewhere, have repeatedly confirmed the above observations. These results in the mouse differ markedly from those in the hypophysectomized rat reported by Greer (1949) who found that the ratio approaches approximately 7, and by VanderLaan and Greer (1950) who found that the ratio frequently approaches 1. The relative insensitivity of this ratio to hypophysectomy in the mouse makes it possible to recognize the importance of the binding process as an important factor in the decreased accumulation of I^{131} in hypophysectomized mice.

SUMMARY

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration in male C_3H mice with normal or hyperplastic thyroid glands decreased progressively with increasing time after hypophysectomy. The proportion of the thyroid I^{131} in the thyroxine fraction also decreased rapidly after hypophysectomy in mice fed the stock diet. The thyroid iodide/serum iodide concentration ratio in the mice with hyperplastic thyroids exhibited a decrease during the 13 days after hypophysectomy but remained elevated above 50.

The decreased accumulation of bound I^{131} in the thyroid glands of these hypophysectomized mice did not appear to be due to a decrease in thyroid radioiodide concentration.

Acknowledgement

We are indebted to Dr. Charles C. Congdon for his review of the serial sections of the region of the sella turcica, and to Mr. Donald F. M. Trainor for technical assistance.

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MECHANISMS OF PARTIAL BLOCKADE OF THE STRESS RESPONSE IN RATS BY DIBENAMINE ANALOGUES[1]

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INTRODUCTION

IT IS generally agreed, following the pioneer work of Long and Fry (1945) and Vogt (1945), that physiological dosages of epinephrine will activate the release of adrenocorticotrophin (ACTH) from the adenohypophysis. However, there is considerably less accord as to the mechanism by which epinephrine exerts this effect. Long and his colleagues (Gershberg *et al.*, 1950; McDermott *et al.*, 1950) have presented strong evidence, confirmed by Fortier (1951 b), that injected epinephrine may act directly on the adenohypophysis. Hume and collaborators (Recant *et al.*, 1950) suggest that epinephrine activates the anterior hypothalamus to liberate a humoral substance which, in turn, stimulates the hypophysis. Sayers (1950, 1951) considers that epinephrine increases tissue utilization of cortical steroids and that their depletion from the blood "fires" the ACTH release mechanism. Porter (1952) has recently reported direct evidence that epinephrine activates the posterior hypothalamus and that lesions in this region prevent stress-induced discharge of ACTH. DeGroot (1952) also favors the hypothalamus as the primary site of epinephrine action, for he found that epinephrine would not stimulate lymphopenia in mice with their hypophyseal portal systems damaged.

The hypothesis that reflex secretion of endogenous epinephrine is essential in the natural mechanism by which stress activates ACTH release is confronted by similar lack of agreement. Gordon (1950 a, b) reported a normal response to "alarming" stimuli in rats with demedullated adrenal glands. This was denied by Gershberg *et al.* (1950) and Dury (1950). The controversy as to whether epinephrine is a specific link in the pituitary-adrenal mechanism or whether it acts like other non-specific stresses, is treated in the discussions of recent symposia reviews by Pincus (1950), Sayers (1951), Harris (1952) and Long (1952). Long's group has proposed a

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dual mechanism in which systemic epinephrine is responsible for an initial *autonomic* phase of pituitary stimulation but not for the later *metabolic* phase.

A barrier to the acceptance of a specific role for intrinsic epinephrine is the almost universal failure of attempts with adrenergic blocking agents to interfere with ACTH release. Partial blockades of the mechanism by ergotamine (Ronzoni and Reichlin, 1950) and by Dibenamine (Seifter *et al.*, 1949; Paschkis *et al.*, 1950; Ronzoni and Reichlin, 1950) have been reported. Not only was the degree of blockade slight but there was no attempt made to test whether even the partial effect was actually due to adrenergic blocking properties of the drugs. This latter consideration assumes importance in view of results to be presented below. Moreover, in the hands of other investigators, Dibenamine (Tepperman and Bogardus, 1948; Fortier, 1951 a), ergotamine (Tepperman and Tepperman, 1950; Gershberg *et al.*, 1950) and dihydroergocornine (August and Gubner, 1949) have been completely ineffective in interrupting the stress response. Whereas these negative results are inconclusive, they have failed to lend support for a significant role of endogenous epinephrine in the ACTH release mechanism.

In 1950, Sawyer *et al.*, reported that a new adrenergic blocking agent SKF-501 (N [9-Fluorenyl]-N-ethyl-β-chlorethylamine hydrochloride) was more highly effective than Dibenamine in blocking the neurogenic stimulus for LH (ovulating hormone, gonadotrophin) release from the adenohypophysis of rabbits and rats. SKF-501 has been shown to have 3-10 times as potent adrenergic blocking properties as Dibenamine (Fellows, 1949; Sawyer *et al.*, 1950). In adrenergic-blocking dosages, SKF-501 prevented ovulation in 80 per cent of the rabbits and rats tested. The mechanism of blockade was apparently anti-adrenergic activity since the effectiveness of SKF-501 was not shared by 2-dibenzylaminoethanol (D-ol), a Dibenamine derivative affecting the central nervous system but lacking adrenergic-blocking capacity. It seemed desirable, therefore, to examine the effects of both SKF-501 and D-ol on the stress response. The present study reveals that SKF-501, but not D-ol, largely blocks the stress-response to injected epinephrine in rats as evidenced by effects on the eosinophil count. Interestingly enough, both SKF-501 and D-ol prevent the autonomic phase in response to such non-specific stresses as cold and formalin injection. The results are interpreted as indicating that D-ol interrupts the reflex arc for neurogenic secretion of endogenous epinephrine in response to non-specific stress, and that SKF-501 may function in this manner as well as having the capacity to block the action of epinephrine at its effector site. The results are consistent with the sequence proposed in the dual hypothesis of Long *et al.* (*op. cit.*). Part of the present results, with tentative interpretations somewhat at variance with the present ones, have appeared in abstract form (Sawyer and Parkerson, 1951; Sawyer, 1952).

MATERIALS AND METHODS

The experiments fall into two principal groups: those conducted in North Carolina (Series A) and those in California (Series B). In Series A, male rats of the inbred Vanderbilt (Osborne-Mendel) strain, ranging in weight from 220–360 grams, were used in the hematological study. The rat room was air-conditioned at a summer temperature of about 27° C. The animals were kept in wire mesh cages, not more than three to a cage, and fed *ad lib* on Purina Dog Chow. Lighting conditions were controlled by a time switch to give 14 hours of light per day (Everett, 1948). In Series B, Wistar rats ranging in weight from 150–260 gm. were employed. Lighting and temperature conditions were not rigidly controlled but on the day of each experiment, care was taken that the rats not be disturbed prior to the administration of the controlled stressing stimulus. Other conditions were similar to those in Series A. In both series, the stressing stimulus was applied routinely between 9–10 A.M.

The method employed to assess ACTH release was the eosinophilic response of Thorn *et al.* (1948), and direct eosinophil counts were made with a modification of Randolph's (1944) phloxine-propylene-glycol diluent. A small blood sample was collected from the tail vein into an oxalated depression in a paraffin block. Within a few minutes, in Series A, three WBC pipettes were filled to the 0.1 mark and diluted to the 1.1 mark. They were shaken for about 30 seconds and set aside for at least 15 minutes for staining. After shaking again at a fairly rapid rate for 2 minutes in a mechanical shaker, each of two pipettes was used to fill 4 Spencer Bright-Line chambers, with a 30-second period of shaking between chambers. If the 36-square counts for each of the two pipettes checked within 15%, the third pipette was not used; if not, the average of all three was taken. Most of the Series A eosinophil counts for control rats ranged between 30–80 cells per cubic mm., and repeated counts on individual animals, with a few days interval between bleedings, gave very similar values. These counts are lower than most of those reported heretofore, indicating that strain differences in rat eosinophil levels may exist. Most of the Series B Wistar rat eosinophil counts ranged between 100–300 cells per cubic mm. Seventy-two squares filled from 2 WBC pipettes were counted in the eosinophil determinations and rats were not used whose pre-stimulation level was less than 70/cu. mm.

Three types of stimuli for ACTH release were employed: epinephrine, cold stress, and formalin. Epinephrine (Parke-Davis Adrenalin, 1/1000–1/5000) was injected subcutaneously at a dosage of 0.2 mg./kg. Ten per cent formalin was injected subcutaneously at a constant dosage of 0.3 ml. As a cold stress stimulus, rats were kept for one hour in a refrigerator room at a temperature of 8–10° C., nearly 20° C. less than the rat room temperature. In the cold stress groups, blood samples were taken at 0 and 4 hours, so the stimulus includes handling and bleeding as well as the temperature changes. Control blood samples in the Series A epinephrine group were taken a few days before or after the experimental 4-hour sample, so that the effects of epinephrine were not complicated by additional stress. The Series B eosinophil counts assayed blood samples taken at 0, 1 and 4 hours in both the epinephrine and formalin series, so the handling and bleeding element in the stress stimulus was considerable. Routinely there was a 4-day recovery period between consecutive stress experiments on a given animal, and more than a week was allowed for recovery from SKF-501 or D-ol injection.

Sodium pentobarbital (Nembutal, Abbott), which was employed intraperitoneally in preliminary experiments in Series A, was in the form of the veterinary preparation in an alcohol-propylene-glycol-water vehicle or a 2 per cent solution in Ringer-Locke. The latter solution, which was made up just before use, was warmed to body temperature and injected intraperitoneally at a dosage of 30–50 mg./kg.

The adrenergic blocking agent, SKF-501, was dissolved in a propylene-glycol vehicle at a concentration of 50 mg./ml.; this was diluted for use to 5 mg./ml. with Ringer-Locke solution. Intravenous injections were performed into the lesser saphenous vein below the ankle as described earlier (Sawyer, Everett and Markee, 1949). The dosage adopted for use, 10 mg./kg., is above the MLD: in the present experiments 2 rats receiving this dose died during the following night. This dosage level was chosen because of its demonstrated ability in the rat and the rabbit to block the stimulus for release of pituitary LH without interfering with the release mechanism itself or with the action of hormone on its target organ (Sawyer *et al.*, 1950). Dibenzylaminoethanol was injected intravenously at a concentration of 10 mg./ml. in saline, and the dosage of 15–20 mg./kg. usually induced immediate convulsive muscular activity which lasted about one minute. Thereafter, the rats' motor activity appeared to be depressed.

RESULTS

Stress responses induced by Nembutal, SKF-501, and D-ol. Our first experiments were an unsuccessful attempt, with Nembutal anesthesia, to stabilize the eosinophil count against depression from handling and bleeding. It was hoped that even intravenous injections of SKF-501 might be made under anesthesia without inducing an alarm reaction. Recant *et al.* (1950) and Ronzoni (1950) had reported such a stabilizing effect of the anesthetic. As summarized in Figure 1, our results were completely negative in this respect. Nembutal itself, in confirmation of Ludewig and Chanutin (1947), acted as a stress stimulus leading to a 50% reduction in the eosinophil count after 4 hours. It appeared to make little difference whether the Nembutal was in alcohol-propylene-glycol-water or in Ringer-Locke buffered with sodium carbonate, at room temperature or warmed. Nor did "training" the rats with twice-daily mock injections for a week lessen the stress response.

In spite of the fact that its central excitatory effects are much less severe than those induced by Dibenamine (Sawyer *et al.* 1950), intravenous injection of 10 mg./kg. SKF-501 was followed by a marked reduction in circulating eosinophils (Fig. 1). When the SKF-501 injection was preceded by intraperitoneal Nembutal the decrease was somewhat less, but the number of cases was too small to make the difference significant. Even if it were significant and if SKF-501 were completely ineffective in blocking ACTH release, a 60 per cent decrease in eosinophils is already so great that one could not expect much further reduction in response to cold stress or epinephrine. Inasmuch as the adrenergic blocking capacity of β-chloroethylamine derivatives lasts for days (Nickerson, 1949) we next tested whether the eosinophil count would return to normal by 24 hours after injection of SKF-501. This was observed to be true (Fig. 1), and the finding allowed us to examine the drug's capacity to block the eosinopenic response to stress at that time. Marked eosinopenia was also induced by injection of D-ol, followed within 24 hours by a return to nearly normal levels (Fig. 1). Later results with Wistar rats (Series B) revealed eosinophil counts characteristically higher than normal, 24 hours after injection of either SKF-501 or D-ol.

To demonstrate that adrenergic blockade by SKF-501 does indeed persist for 24 hours after treatment, we injected 3 rats with 5 mg./kg. SKF-501 (only one-half our usual dose) and followed at 24 hours with 0.2 mg./kg. intravenous epinephrine. This dosage of epinephrine was found in preliminary experiments to be in the realm of twice the LD_{100}, and in the present series it killed 2 control rats and 3 rats injected 24 hours earlier with 15 mg./kg. D-ol, in less than 5 minutes, all with symptoms of pulmonary edema. The 3 SKF-501-epinephrine-treated rats showed no such symptoms, and all survived. Thus SKF-501, at a dosage only half as great as that used in our ACTH experiments, protects at 24 hours against a twice-lethal dosage of epinephrine, whereas D-ol, at full dosage, lacks the capacity to block epinephrine effects.

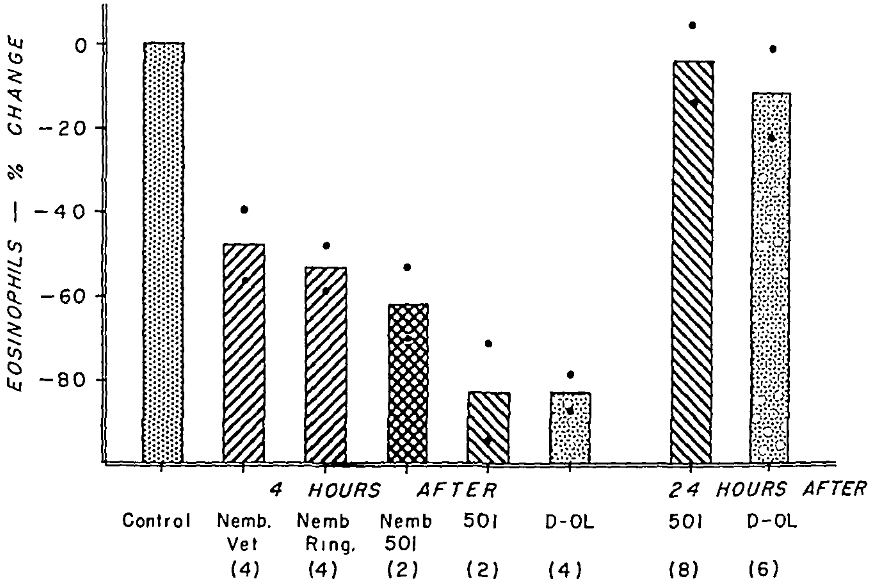

FIG. 1. Results of preliminary experiments in Series A. The effects of Nembutal, SKF-501, and D-ol on circulating eosinophils are expressed as percentages of the 0-hour counts. The figures in parentheses represent the number of rats employed in each group. Each set of dots limits the standard error of the mean.

Effects of SKF-501 and dibenzylaminoethanol on the 4-hour eosinophil responses to epinephrine and to cold stress. The results are summarized in Figure 2. Each of the columns represents data on at least 8 rats except the last one in which only 2 animals were employed. However, only 17 rats were used in this whole series, and as far as possible the same rats were subjected to a given stressful stimulus and attempted blockade of that stimulus. It is apparent that either subcutaneous epinephrine or exposure to cold stimulates a marked decrease in the number of circulating eosinophils, about a 70 per cent depression in either category. The eosinopenia stimulated at 24 hours after SKF-501 injection, though still considerable in the cold-stressed or epinephrine-treated rats, is significantly reduced.

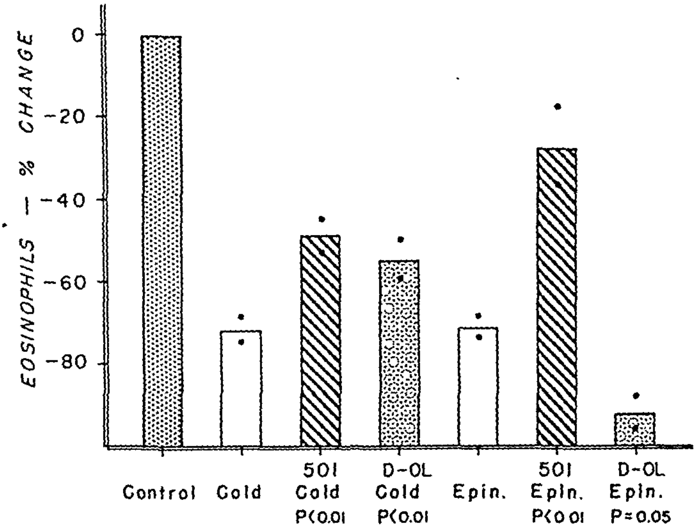

FIG. 2. Effects of "blocking" agents on the 4-hour eosinophil response to cold stress and to epinephrine (Series A). The P values for the differences between stressed and stressed-"blocked" animals were calculated from eosinophil-count data, not from the percentage-change columns shown in the figure.

$P < 0.01$ in either case between the eosinophil levels of SKF-501-treated and non-treated groups. The failure to respond 24 hours after SKF-501 is probably not attributable to the initial non-specific stress of SKF-501 itself, for 4 controls each gave a normal eosinophil response to epinephrine or cold stress 24 hours after epinephrine or cold stress, respectively. Interestingly enough, however, D-ol "blocked" the response to cold stress about as effectively as SKF-501 without preventing epinephrine-induced eosinopenia at all. The degree of eosinopenia in the D-ol-epinephrine group was, if anything, greater than in the controls lacking D-ol.

Effects of SKF-501 and dibenzylaminoethanol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin. These experiments were undertaken after the appearance of Long's dual hypothesis (McDermott *et al., op cit.*) to determine whether the autonomic phase of pituitary stimulation might be prevented by adrenergic blockade. The results are summarized in Figure 3. Pre-treatment with SKF-501 but not with D-ol completely eliminated the eosinopenia at 1 hour after epinephrine. The rats pretreated with D-ol again showed a response to epinephrine, perhaps more marked than the controls. The 4-hour results confirm the earlier findings with epinephrine, except that the SKF-501 column reflects the added stress of handling and bleeding at 0-hour and 1-hour collectively.

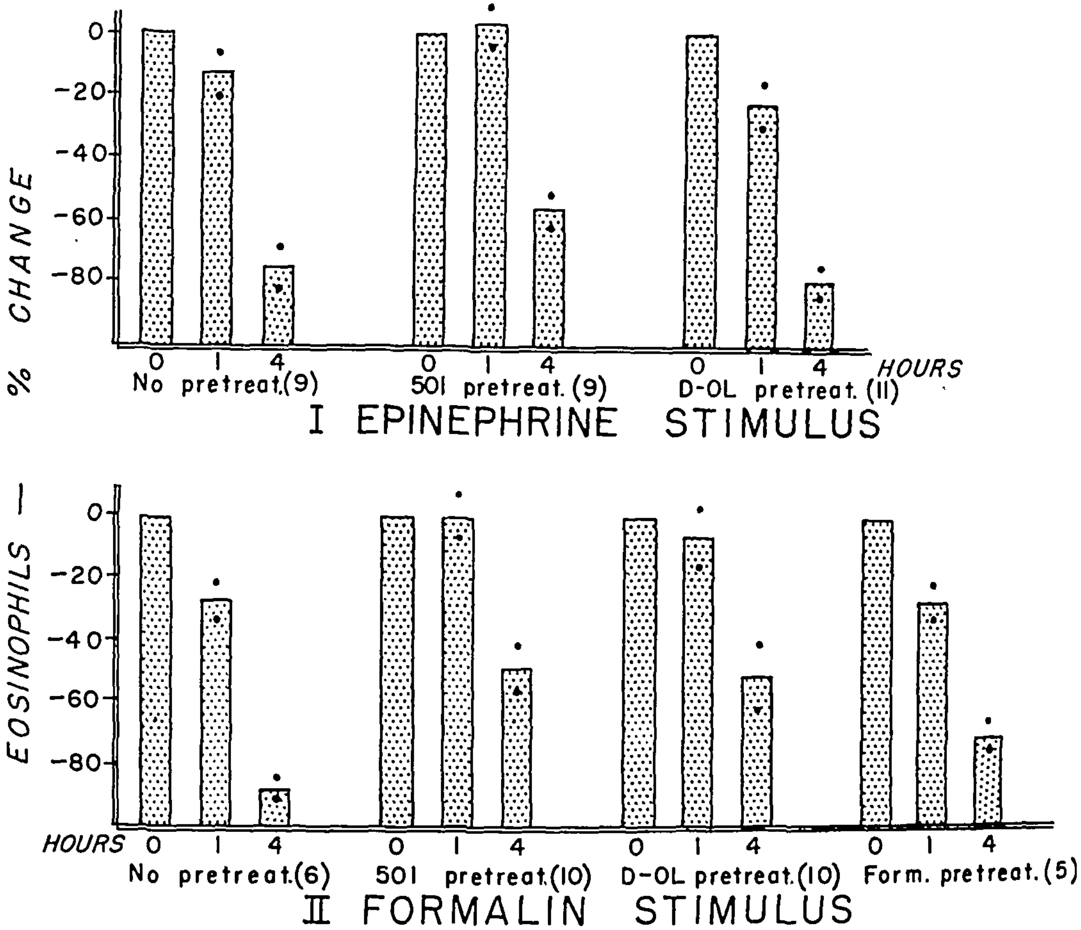

FIG. 3. Effects of pretreatment with SKF-501 and D-ol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin (Series B). In group I, epinephrine was injected subcutaneously, 24 hours after treatment with SKF-501 or D-ol. In group II, formalin was injected subcutaneously 24 hours after treatment with SKF-501, D-ol or formalin itself. Figures in parentheses represent numbers of rats employed in each group.

Formalin (Fig. 3) induced an eosinopenia at 1 hour at least as great as that stimulated by epinephrine and the effect was not lessened by formalin stress on the preceding day. However, both SKF-501 and D-ol appeared to prevent this 1-hour response to formalin and to diminish the 4-hour response in the same degree as they had lessened the response to cold.

DISCUSSION

It seems clear from the present results that β-chloroethylamine adrenergic blocking agents may partially prevent stress-induced eosinopenia by more than one mechanism. SKF-501 and dibenzylaminoethanol exert similar degrees of "blockade" against formalin or cold stress but differential effects against epinephrine. The blockade of formalin and cold stress by D-ol must be exerted by some means other than adrenolytic action. The fact that epinephrine is effective in stimulating a normal eosinopenia in spite of D-ol indicates that the final stages in the eosinopenic mechanism are not disturbed by chloroethylamine derivatives. Therefore, the failure of cold or formalin to induce a full-fledged eosinopenia after D-ol or SKF-501 cannot be attributed to the inability of the effector end of the eosinopenic mechanism to respond. Presumably the adrenal cortex and the pituitary ACTH release mechanism are intact.

While lacking the capacity to counteract epinephrine, D-ol blocks the autonomic phase of eosinopenia in response to cold or formalin. It seems most likely that it does so by interfering in some way with the stimulus for release of endogenous epinephrine. In this capacity its action may be analogous to those of barbiturates which were reported by Sayers and Sayers (1946) and Gellhorn (1949) to interfere with adrenal ascorbic acid depletion and lymphopenia, respectively, in the rat after generalized stress but not after injection of epinephrine. D-ol does, in fact, appear to be a depressant after a brief period of excitation of the central nervous system.

SKF-501 exerts a similar though less marked depressant effect, and its primary action against formalin and cold stress may be the same as that exerted by D-ol. However, SKF-501 has, in addition, very potent adrenolytic properties and these are credited with its blockade of epinephrine-induced eosinopenia. There is no evidence that epinephrine was responsible for any of the 4-hour eosinopenia encountered after cold, formalin or epinephrine injection in SKF-501-treated animals. Where non-specific stress was least (i.e. epinephrine injection without bleeding), the 4-hour eosinopenia was least evident.

It is significant that SKF-501 does not prevent the 4-hour eosinopenia from cold or formalin any more effectively than does D-ol. This must mean that under either SKF-501 or D-ol, neither cold nor formalin stimulates a systemic discharge of epinephrine and that the eosinopenia which is achieved is effected via ACTH release from another mechanism, e.g., the "metabolic" phase. The results allow one to make a rough calculation of the relative part played by epinephrine in both autonomic and metabolic phases of eosinopenia. With epinephrine eliminated via blockade, the non-specific metabolic effects induced about zero % and 50% eosinopenia at 1 and 4 hours, respectively, whereas the combined autonomic and meta-

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NOTES AND COMMENTS

ADSORPTION OF OXYTOCIN UPON OXIDIZED CELLULOSE

In the course of using oxidized cellulose for selective adsorption and elution of adrenocorticotrophic hormone, by the method of Astwood *et al.* (1951), it was noted that the oxytocic contaminant of the ACTH was removed along with adrenocorticotrophic activity, but unlike the corticotrophic factor it was not appreciably eluted with 0.1 N HCl (1952). Dumm (1952) has shown that the antidiuretic factor can be quantitatively adsorbed upon oxycellulose and completely eluted therefrom by methods similar to that utilized for ACTH (1).

The present communication deals with the attempts to selectively adsorb and elute the oxytocic principle of the posterior pituitary gland, using oxidized cellulose as the adsorbent.

METHODS AND RESULTS

In the initial experiment 100 mg. of lyophilized posterior pituitary gland powder was extracted with 100 ml. of 0.25% acetic acid by boiling for 1 minute and filtered. To the extract was added 2 gm. oxidized cellulose (Tennessee-Eastman, 10–12% Carboxyl) previously washed with 1 N HCl, then H_2O and finally 0.1 N HAC. The pH of the mixture was 3.3. The mixture was stirred at room temperature and aliquots removed at various times for determination of oxytocin content by the rooster blood pressure drop method (1939). A similar solution kept at room temperature with stirring for 24 hours but without oxycellulose served as a control and showed no loss in activity due to oxidative changes which may have occurred during the stirring. Results are summarized in Table 1.

TABLE 1

Duration of stirring with oxycellulose	Oxytocin Present (U.S.P. units)	Per Cent Oxytocin Removed from Solution
0	150	0
1 hour	80	53
2 hours	38	75
4 hours	42	72
24 hours	39	75

Attempts to elute the oxytocic factor from the oxycellulose with 0.1 N HCl and 1 N HCl resulted in a recovery of only 30% of the activity.

The optimum amount of oxycellulose necessary to obtain maximum adsorption was determined by stirring 100 ml. posterior pituitary extract containing 100 U.S.P. units of oxytocic activity with varying quantities of oxidized cellulose for a period of 5 hours at pH 3.3. Table 2 presents the data obtained.

TABLE 2

Amount Oxycellulose	Per Cent Adsorption
50 mg	34
200 mg	42
500 mg	55
2000 mg	72

The large amounts of oxycellulose necessary to obtain the maximum adsorption of oxytocin when extracts of posterior pituitary gland were used, were somewhat unexpected in view of the small quantities necessary to secure a similar amount of adsorption of the oxytocic contaminant from ACTH solution. Table 3 indicates this difference. The amount of oxycellulose employed in each case was 200 mg.

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TABLE 3

Fraction	Oxytocic content before adsorption U.S.P. units	Oxytocic content after adsorption U.S.P. units	Per Cent Adsorption
Crude ACTH	4200	1100	74
Crude ACTH	4200	1000	76
Crude ACTH	675	160	76

DISCUSSION

These data indicate that a maximum adsorption of about 76% of the oxytocic factor can be obtained by the use of oxycellulose. However it is to be noted that much smaller amounts of oxycellulose sufficed to remove the oxytocic factor from solutions of ACTH contaminated with oxytocin than from solutions prepared by extraction of the posterior lobe of the pituitary gland.

From the data presented it is not possible to explain the inability to entirely elute the oxytocic factor from oxycellulose using 0.1 N HCl and 1 N HCl, which observation contrasts with those of Dumm who found that the antidiuretic factor was completely eluted with 0.1 N HCl. Experiments are now in progress to determine whether buffered salt solutions may accomplish recovery of the oxytocin from the oxycellulose column. The possibility of destruction of the oxytocin during the stirring with oxycellulose by surface denaturation cannot be ignored, although this possibility is remote in view of Dumm's (1952) findings that the antidiuretic factor can be quantitatively adsorbed onto and eluted from oxycellulose columns.

It is also difficult to explain the observation that only about 75% of the oxytocin is adsorbed onto the oxycellulose even when very large amounts of oxycellulose are used.

The results obtained were utilized to accomplish removal of the major portion of oxytocic contamination from the adrenocorticotropic factor isolated from whole hog pituitary glands, and seems to offer a rather effective method for separation of the bulk of oxytocic contaminant from the adrenocorticotrophic hormone.

Alginic acid (Kelacid*) was found to have adsorptive properties similar to oxycellulose in its power to adsorb oxytocin and ACTH.

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* Kelco Co., New York City.

HIGH PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE

Among the many compounds tested for progestational activity, few have exhibited greater biological potency than progesterone (Ehrenstein, 1948).

Meystre *et al.* (1948) have shown 11-dehydroprogesterone to possess a threefold greater potency than progesterone in the Clauberg test. 17-methylprogesterone A has twice the activity of progesterone using the Corner-Allen assay method (Heuser *et al.*, 1950). Ehrenstein (1944) described the degradative synthesis of 19-norprogesterone from strophanthidin. This material was a resinous substance believed to be a mixture of stereoisomers. In a study of its physiological activity, using the Corner-Allen technique, it was reported to be "fully as active as progesterone, perhaps even more so" (Allen and Ehrenstein, 1944). The synthesis of 19-norprogesterone as the pure crystalline compound by Miramontes *et al.* (1951) made possible the present investigation of its biological activity in the rabbit.[1]

MATERIALS AND METHODS

Rabbit Bioassays:

(a) *Clauberg Test* (Clauberg, 1930). Female New Zealand white rabbits, weighing 600–800 gm., were injected subcutaneously daily with 10 mcgm. alpha estradiol in 0.5 ml. corn oil for 6 days. Following this, the progestagen was injected daily subcutaneously in corn oil solution for 5 days. Twenty-four hours after the last injection the animals were killed. Uteri were weighed and fixed in 4% formalin, sectioned at 8 microns and stained with hematoxylin and eosin.

(b) *Corner-Allen Test* (Corner and Allen, 1929). Sexually mature female New Zealand white rabbits, isolated for 4 weeks were injected subcutaneously with the test compound for 5 days following bilateral ovariectomy and biopsy of the right uterine horn. Approximately 24 hours after the last injection, the animals were killed and the uteri were treated as in (a). The uterine biopsies were taken for histological examination and only those animals were used in which the uterine biopsy showed an estrogen-stimulated endometrium. In each bioassay method, progestational proliferation was rated according to the standard scale of McPhail (1934).

RESULTS AND DISCUSSION

The activity of 19-norprogesterone was compared with that of progesterone in a series of experiments, using the Clauberg method. Table 1 presents data from two of the series.

TABLE 1. PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE USING THE CLAUBERG TEST

Series	No. of rabbits	Total dose (mcgm.)*	Progestational proliferation	
			Progesterone	19-norprogesterone
I	3	250	+3, +4, +4	—
	3	500	+4, +4, +3	—
	3	62.5	—	+3, +3, +4
	3	125.0	—	+4, +4, +4
II	3	125	0, 0, 0	—
	3	250	+3, +3, +3	—
	2	31.25	—	+3, +2
	3	62.5	—	+4, +4, +4

* Administered in 1.0 ml. corn oil solution daily over 5 days.

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[1] We are indebted to Chemical Specialties Co. Inc., and Syntex, S.A., for the steroid compounds used in these investigations.

subjected to ether anesthesia during the placing of the needle into the jugular vein but were thereafter kept in activity-restriction cages without anesthesia until death. The dose of cortisone (4 mg. per rat per day) was selected from other experiments (Ingle, Nezamis and Morley, 1951) in which this dose was found to be optimal for sustaining the resistance of the adrenalectomized rat to the severe stress of muscle work.

The data are summarized in Table 1. Cortisone did not significantly affect the resistance of the rat to histamine under these conditions although it is known to suppress the inflammatory response to injury in the normal rat. The amount of histamine required to cause death in the rat under these conditions is remarkable.

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RELATIVE INEFFECTIVENESS OF ELECTRICAL STIMULATION OF THE CERVIX FOR INDUCING PSEUDOPREGNANCIES IN THE MOUSE

The occurrence of pseudopregnancy in the female rat after mating with a vasectomized male was reported by Long and Evans (1922). They also induced this condition by mechanical stimulation of the cervix with glass rods. Parkes (1926) described similar responses in the mouse. Electrical stimulation of the cervix of the rat, by means of small electrodes and a simple induction coil as reported by Shelesnyak (1931) produced a larger percentage of pseudopregnancy than mechanical irritation. This is a report of the relative ineffectiveness of electrical stimulation in producing pseudopregnancy in the mouse.

In a series of experiments requiring pseudopregnant C_3H strain mice, electrical stimulation of the cervix was used. Thirty-six C_3H mice, weighing between 24 and 26 grams, with regular 4–5 day oestrous cycles were selected at the proestrus stage. Intra-vaginal electrical stimulation with Faradic current of approximately 75 volts, peak (Phipps and Bird inductorium, A70-720) was applied blindly to the cervical area for 10 to 15 seconds. No attempt was made to visualize the os cervix for precise placing of the electrodes since in the rat this has not been necessary. Only 8 of the 36 mice became pseudopregnant (averaging 11 days duration) and in 26 of the failures which were stimulated a second time at the next proestrum only 4. Thus out of 62 attempts there were 50 failures. A second group of 40 C_3H mice was treated, using an electronic stimulator, which generated spike waves similar to the inductorium, 105 volts 50 cycles per second. Only 10 of the 40 became pseudopregnant. In the combined series of C_3H mice, 76 females where stimulated at proestrous or early oestrous and after 102 attempts, 22 pseudopregnancies were produced.

To determine whether this low percentage of pseudopregnancies following electrical stimulation of the cervix was a strain or a species difference, the same procedure was carried out on 58 Swiss mice (of which 16 were stimulated on two or more successive cycles). In 22 instances, the stimulus was through the use of the induction coil; in 52, with the electronic stimulator. In the combined group of 58 Swiss mice in which 75

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stimulations were made the number of pseudopregnancies which resulted was 19; and thus the response was similar to that of the C_3H strain.

It appears that a species difference exists between the mouse and the rat in respect to the response of the female to electrical stimulation of the cervical areas during proestrun. In the rat over 90% become pseudopregnant (Shelesnyak, 1931), while in the mouse less than 26% do.

Acknowledgement

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THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.

THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

THE LAURENTIAN HORMONE CONFERENCE

The Laurentian Hormone Conference of the AAAS will hold its 1953 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 6–11. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Mass., for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than *May 15* in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. *Nervous system-hormone interrelationships*
  The central nervous system and stress-induced eosinopenia.
    Dr. R. W. Porter, Univ. of Calif. School of Medicine at Los Angeles
  Studies of brain metabolism and electrical activity in relation to adrenocortical physiology.
    Dr. Hudson Hoagland, The Worcester Foundation for Experimental Biology
  Effects of hormones on cerebral function.
    Dr. D. M. Woodbury, Univ. of Utah School of Medicine

II. *Thyroid Hormone physiology and biochemistry*
  Triiodothyronine in relation to thyroid physiology.
    Drs. J. Gross and R. Pitt-Rivers, National Inst. for Medical Research, London, and State Univ. of New York Medical Center
  Metabolic effects of thyroid hormones *in vitro.*
    Drs. H. A. Lardy and G. Feldott, Univ. of Wisconsin School of Medicine

III. *Comparative endocrinology*
  Endocrine mechanisms in the life of insects.
    Dr. D. Bodenstein, U. S. Army Chemical Center, Maryland
  Hormones produced by neurosecretory cells.
    Drs. Ernst and Berta Scharrer, Univ. of Colorado Medical Center

IV. *Protein Hormones*
  The preparation and chemistry of crystalline insulin.
    Dr. R. G. Romans, Connaught Medical Research Laboratory
  The chemistry of ACTH.
    Drs. E. E. Hays and W. F. White, The Armour Research Laboratories

V. *The role of hormones in blood and blood-forming organs*
  Endocrine factors and radiation-induced lymphoid tumors of mice.
    Dr. Henry S. Kaplan, Stanford Univ. School of Medicine
  Endocrine influences upon the formed elements of blood and blood-forming organs.
    Dr. Albert S. Gordon, Washington Square College, New York Univ.

VI. *Aspects of Clinical Endocrinology*
  Some problems related to ovarian function and to pregnancy.
    Dr. B. Zondek, Rothschild Hadassah Univ. Hospital, Jerusalem
  Clinical studies on electrolyte and fluid metabolism.
    Dr. Rolf Luft, Serafimerlasarettet, Stockholm
  Endocrine-metabolic studies in man.
    Dr. J. W. Conn, Univ. of Michigan School of Medicine

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THE RESPONSE OF THE SUCCINOXIDASE SYSTEM OF RAT LIVER HOMOGENATES TO A SINGLE DOSE OF THYROXINE

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INTRODUCTION

THE *in vivo* effect of thyroxine on the metabolic rate of whole animals, on the oxygen consumption of isolated tissues and on the activity of various enzyme systems has been reviewed by Barker (1951). The mechanism of thyroxine action has been studied most generally in chronically thyroxinized animals. Barker, Kiely and Lipner (1949) have indicated the time lag between the administration of thyroxine and the first measurable increase in the oxygen consumption of the whole animal. Barker and Klitgaard (1952) have demonstrated the unequal response of the tissues of rats to thyroxine. The earliest response elicited occurred 6 hours following a single injection of thyroxine, and liver slices responded in 12 hours. An understanding of the mechanism of thyroxine action involves the elucidation of the cellular processes responding to thyroxine prior to the changes which have been recorded.

This study is concerned with the time response of the liver succinoxidase system of thyroidectomized rats to thyroxine. An analysis of the succinoxidase system of cell granules was performed simultaneously with the study of this enzyme system in rat whole liver homogenates.

METHODS

Male rats of the Sprague-Dawley strain, weighing 300–350 grams, were used in all the experiments included in this study. These animals were maintained on a 5-hour feeding regimen (Barker, 1949) and were usually post-absorptive at sacrifice. The animals were exsanguinated under ether anesthesia by withdrawing 8 to 10 ml. of blood

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[1] Baxter Laboratories Fellow 1951–52.

from the abdominal aorta into an oxalated syringe. The liver was then perfused through the portal vein with 100 ml. of cold Krebs-Ringer solution. A blotted, weighed portion of liver was minced with scissors and homogenized with 2 volumes of 0.25 M sucrose solution in an all glass homogenizer (Potter and Elvehjem, 1936). The homogenate was centrifuged for 5 minutes at 600× gravity and the supernatant decanted into a graduated cylinder. The residue was rehomogenized in 2 volumes of the sucrose solution and then added to the supernatant in the cylinder. The final dilution was such that 1.0 gram of liver was contained in 10 ml. of homogenate suspension.

The fractionation procedure employed was essentially that described by Schneider and Hogeboom (1950).[2] The entire procedure, from the time the liver was excised until the enzyme assay was performed, was carried out at a temperature of 2° to 4° C.

The succinoxidase activity of rat liver homogenates was studied in 5 groups of rats: *normal controls, thyroidectomized controls and 3 groups of thyroidectomized rats* given 6.12 mg./kg. of thyroxine (in 0.02 N NaOH, 0.9 per cent NaCl solution) at 12, 24, and 96 hours previous to sacrifice. The method employed for the assay of succinoxidase is that described by Potter (1949) with the following 2 modifications: (1) it was found unnecessary to perform this assay at 2 enzyme concentrations and (2) determinations of endogenous oxygen consumption were omitted. A series of preliminary experiments indicated no difference in metabolism as calculated from the 2 enzyme concentrations and no endogeneous oxygen consumption. The metabolic activity is expressed as μl. of oxygen consumed per mg. equivalent of liver homogenate per hour.

RESULTS

A summary of the data indicating the effect of thyroxine on the succinoxidase system of rat liver homogenates and cell fractions is presented in Table 1. This system in the thyroidectomized rat liver homogenate is

TABLE 1. THE MEAN SUCCINOXIDASE ACTIVITY OF RAT LIVER HOMOGENATES AND CELL FRACTIONS FOLLOWING A SINGLE INJECTION OF THYROXINE TO THYROIDECTOMIZED RATS

	Normal	Tx	Tx	Tx	Tx
			12 hr. T	24 hr. T	96 hr. T
No. of animals	12	13	7	7	10*
Homogenate†	22.5	20.6	24.1	23.0	28.8
S.D.	±1.85	±2.55	±3.62	±1.82	±2.90
Nuclear	4.2	4.5	3.9	4.9	3.2
S.D.	±1.59	±2.10	±2.10	±2.30	±0.42
Mitochondrial	14.3	12.8	15.6	13.1	22.1
S.D.	±2.4	±0.98	±1.2	±0.6	±0.64
Supernatant	2.0	1.8	2.9	2.5	2.4
S.D.	±0.70	±0.84	±0.74	±0.58	±0.56
Sum of Fractions	20.5	18.1	22.4	20.5	27.7

* The livers of 8 animals were used in the fractionation study.

† The means represent μl O_2/mg. wet weight liver or equivalent/hr.

Abbreviations: Tx = thyroidectomized. T = number of hours after a single subcutaneous injection of thyroxine on 6.12 mg./Kg. level.

S.D. = estimated standard deviation of the individual observations from the mean.

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[2] The fractions were prepared in an International Refrigerated Centrifuge, Model PR-I.

approximately 8% less active than in normal controls. Administration of thyroxine resulted in elevation of this level of activity, the 12-hour thyroxinized group showing an increase of approximately 7%, the 24-hour group of approximately 2% and the 96-hour group of approximately 28%.

The activity of this enzyme system in mitochondrial fractions varied with the thyroid status of the animal in a manner similar to that described for the whole homogenate. The nuclear and supernatant fractions possessed succinoxidase activities which, despite alterations in thyroid status of the animals, showed little change.

It should be emphasized that the fractions were not pure preparations since the nuclear fraction was found, by microscopic observation, to be contaminated with small quantities of intact cells, connective tissue, cellular constituents (which were not characterized) and erythrocytes. The mitochondrial or large granule fraction was contaminated to some extent with small granules and occasionally with erythrocytes and nuclei. The final supernatant fraction consisted of the microsomes or small granules, and the soluble constituents of the cell.

Statistical analysis of the data in Table 1 for the whole homogenate succinoxidase activity is presented in Table 2. The data for the fractions were also analyzed statistically. No further information than that obtained from the whole homogenate analysis was discernible. This latter analysis has, therefore, not been included.

TABLE 2. STATISTICAL ANALYSIS OF RAT LIVER HOMOGENATE SUCCINOXIDASE

	No. of animals	Mean μl. O_2/mg. homogenate/hr.	S.D.	t value	Level of significance
					%
I Normal control	12	22.5	1.83		
II Tx	13	20.6	2.55		
III Tx 12-hour T	7	24.1	3.62		
IV Tx 24-hour T	7	23.0	1.83		
V Tx 96-hour T	10	28.8	2.90		
I vs. II				2.15	4.69
I vs. III				1.09	28.66
I vs. IV				0.57	55.64
I vs. V				5.95	0.00
II vs. III				2.27	3.36
II vs. IV				2.42	2.74
II vs. V				7.08	0.00

Abbreviations same as Table 1.

From Table 2 it is apparent that the difference in activity of the succinoxidase system of liver homogenates of normal controls as compared with thyroidectomized rats is significant at the 5% level of confidence. There are no significant differences between the activity of the normal controls and that of the 12-hour or 24-hour thyroxinized animals. The difference in activity between the normal controls and the 96-hour thyroxinized rats is highly significant.

The mitochondrial fractions contributed the following percentages to the whole homogenate succinoxidase activities: normal, 63.4; thyroidectomized, 61.9; 12-hour thyroxinized thyroidectomized, 63.7; 24-hour thyroxinized thyroidectomized 57.2; 96-hour thyroxinized thyroidectomized 75.0. The mitochondrial fraction is primarily responsible for the succinoxidase activity of the cell, since the total recovered activity of the fractions was 91.2, 92.6, 92.4, 89.3 and 94.4 per cent for these groups respectively.

DISCUSSION

The conditions for the assay of the succinoxidase system require that the limiting enzyme in the system: succinic dehydrogenase: cytochrome-c: cytochrome oxidase be the succinic dehydrogenase. Tipton and Nixon (1946) have demonstrated that the cytochrome oxidase system of liver homogenates prepared from normal, thyroidectomized or thyroxinized rats is present in great excess over the succinoxidase system. Schneider, Claude and Hogeboom (1948) have indicated that cytochrome-c is present in excess in the livers of normal rats. However, Drabkin (1950) found that the cytochrome-c content of rat liver diminishes after thyroidectomy and increases in hyperthyroidism. The addition of exogenous cytochrome-c in the assay obviates the possibility of this enzyme being present in deficient quantities. The requirement for exogenous cytochrome-c by the thyroidectomized rat liver succinoxidase system appears to diminish with time after thyroxinization.[3]

Schneider and Hogeboom (1950) have found that the succinoxidase system is primarily contained in the mitochondrial fraction of the rat liver. This localization is unaltered by modification of thyroid status (Table 1). The succinoxidase activity present in the nuclear and supernatant fractions probably indicates the difficulty in achieving preparations uncontaminated by mitochondria.

The response of the thyroidectomized rat liver succinoxidase system to thyroxine may be described as a triphasic response involving return to the control level of activity, plateau at this level, followed by further increase in activity. The first phase of the response may be due to reactivation of enzymes already present but inactive. The second and third phases are probably related. The plateau may represent the time required for the synthesis of additional enzyme. The further increase in activity probably reflects the increasing concentration of enzymes.

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[3] Unpublished data.

SUMMARY

The activity of the succinoxidase system of rat liver homogenates has been studied in normal control and thyroidectomized rats and compared with that of thyroidectomized rats 12, 24 and 96 hours following the administration of thyroxine.

Under all the conditions studied the succinoxidase system was predominantly present in the mitochondrial fraction. The latter was prepared by differential centrifugation of rat liver homogenates suspended in isotonic sucrose.

The liver homogenate succinoxidase activity of thyroidectomized rats returns to control levels within 12 hours following thyroxine administration and remains at this level for an additional 12 hours before any further increase in activity occurs. The implications of this finding are discussed.

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THE INFLUENCE OF THE ADRENAL CORTEX ON AEROBIC GLYCOLYSIS BY NORMAL AND MALIGNANT RAT LYMPHOID TISSUE[1]

SIDNEY ROBERTS AND ABRAHAM WHITE[2]

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THE regulatory influence of the adrenal cortical secretions on the structure and function of normal lymphoid tissue has been demonstrated by White and Dougherty (cf., White, 1947–48). In addition, a number of studies have indicated that the growth of lymphoid tumors may also be under the control of the adrenal cortex (Heilman and Kendall, 1944; Murphy and Sturm, 1944; Sturm and Murphy, 1944; Law and Speirs, 1947; Diller, Beck, and Blauch, 1948; Pearson, Eliel, and Rawson, 1950; Ingle and Nezamis, 1951; Emerson, 1951).

These observations suggested that the metabolic behavior of normal and malignant lymphoid tissue might be influenced by variations in pituitary-adrenal cortical activity. Accordingly, investigations were undertaken of the influence of the adrenal cortex on the respiratory and aerobic glycolytic activity of normal and malignant rat lymphoid tissue, *in vitro*. These metabolic processes in normal lymphoid tissue were unaffected by the adrenal cortical secretions. However, surviving lymphosarcoma tissue from rats pre-treated with adrenal cortical hormones or ACTH exhibited an increased rate of glucose utilization. This was not accompanied by a change in lactate accumulation or in total oxidative metabolism in the tumor tissue as a result of hormone treatment.

METHODS

The animals supplying normal and malignant lymphoid tissue for the *in vitro* investigations were adult male Sprague-Dawley rats, weighing 250–300 gm. All animals were maintained on Purina laboratory chow and drinking water *ad libitum*, with a weekly supplement of fresh lettuce.

Bilateral adrenalectomy, where performed, was carried out via the dorsal approach 4 to 8 days before autopsy. The animals were then given 1 per cent NaCl to drink instead of tap water. Hormone-treated rats were injected intraperitoneally with ACTH

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[1] Aided by grants from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council, from the Cancer Research Coordinating Committee of the University of California, and from the National Cancer Institute of the National Institutes of Health, Public Health Service.

[2] Present address: College of Medicine, Yeshiva University, 270 Park Avenue, New York 17, New York.

(Actarmour) or ACE (Wilson's aqueous adrenal cortical extract) 2 hours before tissue sampling. The dose of ACTH employed was equivalent to 4 mg. of the Armour standard, LA-1-A, per 100 gm. body weight. Usually, 2 ml. of ACE was the dose used per rat.

The tissues studied included normal spleen, thymus, and mesenteric lymph nodes,[3] and a transplantable lymphosarcoma maintained by subcutaneous passage in Sprague-Dawley rats. This tumor had originally been produced and carried in Wistar animals.[4]

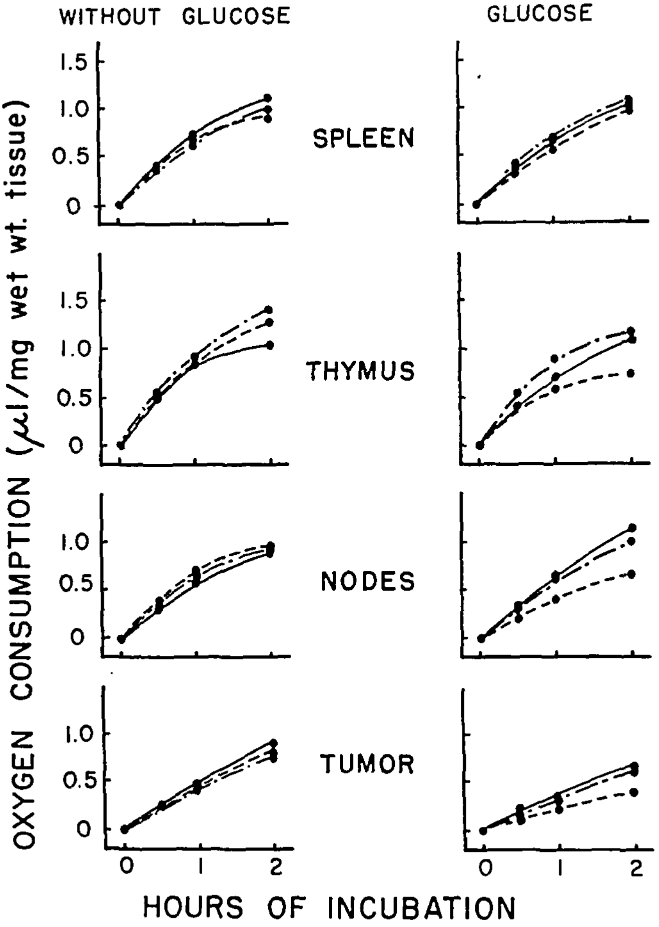

FIG. 1. The influence of the adrenal cortex on the respiration of normal rat lymphoid tissue and a transplantable lymphosarcoma. Solid lines represent tissue obtained from control animals; dotted lines, adrenalectomized; dashed lines, hormone-injected. Each point is the average of 8 to 15 determinations.

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[3] "Mesenteric lymph nodes" refers to the mesenteric chain of nodes dissected free of fat and connective tissue prior to mincing.

[4] We are indebted to Dr. Lloyd Law of the National Institutes of Health for supplying us with this tumor. Histological confirmation of the lymphomatous character of the tumor in Sprague-Dawley recipients was obtained through the generous assistance of Dr. B. G. Lamson, Department of Pathology, School of Medicine, University of California at Los Angeles.

Surviving tissues were obtained from animals which had been exsanguinated from the abdominal aorta while under light nembutal anesthesia. These tissues were rapidly removed, chilled, and minced free-hand with a single-edge razor blade. Approximately 100 mg. of tissue were incubated in 2 ml. of incubation medium at 38° C. Respiration studies were carried out in Krebs-Ringer-phosphate buffer in Warburg flasks which had been gassed with 100 per cent oxygen. In some instances glucose was added to the medium (0.15%), and glucose utilization measurements were made. A number of metabolic studies were conducted in rat serum with a constant stream of 95% oxygen, 5% carbon dioxide passing through the flask. The serum was obtained about one hour before incubation from rats similar to those supplying normal tissue. The glucose content of this serum was approximately 130 to 160 mg. per 100 ml.

After varying periods of incubation, the flask contents were centrifuged at high speed. Aliquots of the supernatant were analyzed for carbohydrate by the anthrone procedure after protein precipitation with trichloroacetic acid (Morris, 1948). Lactate was determined by the method of Barker and Summerson (1941) and amino acid nitrogen by the method of Frame, Russell, and Wilhelmi (1943) on tungstic acid filtrates of the medium.

RESULTS

1. *Respiration of normal and malignant lymphoid tissue*

Comparison was made of the respiration rates of normal and malignant lymphoid tissue minces obtained from adrenalectomized, hormone-injected, and control rats. Samples of spleen, thymus, and mesenteric lymph nodes were obtained from non-tumorous animals of the same age, weight, and dietary history as the rats supplying the lymphosarcoma tissue. The results obtained (Fig. 1) are expressed in terms of µl. oxygen consumed per mg. wet weight of tissue during the two-hour incubation period. In several instances, tissue water determinations were made. The Q_{O_2} values calculated on the usual dry tissue weight basis were substantially in agreement with the oxygen consumption values depicted.

It will be noted that both normal and malignant lymphoid tissues exhibited similar rates of respiration in the absence of glucose in the medium. The addition of glucose to the medium (0.15%) appeared to have no significant effect on the oxygen consumption of normal lymphoid tissue, but depressed the respiration of tumor minces. Hormone treatment (ACE or ACTH) uniformly had no effect on the oxygen consumption of normal or malignant tissues. Adrenalectomy tended to depress respiration only when glucose was present in the medium.

2. *Glucose disappearance and lactate accumulation*

The aerobic disappearance of glucose in the presence of surviving normal and malignant lymphoid tissue was studied in phosphate buffer fortified with glucose (0.15%) under an atmosphere of 100% oxygen and also in a rat serum medium under an atmosphere of 95% oxygen, 5% carbon dioxide. The effect on glucose utilization of pretreatment of the rats with

ACE or ACTH, or of previous adrenalectomy, was also followed.

The results obtained in serum and buffer were similar. Figure 2 and Table 1 show the effect of the adrenal cortical secretions on aerobic glucose utilization by normal lymphoid tissues and by lymphosarcoma tissue in phosphate buffer and serum, respectively. Adrenalectomy, ACE and ACTH were without effect on glucose utilization by normal lymphoid

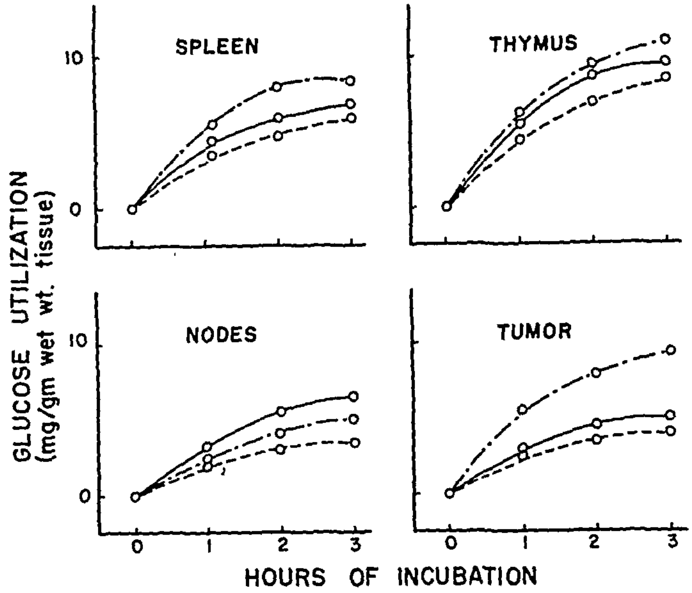

Fig. 2. The influence of the adrenal cortex on the aerobic utilization of glucose by normal rat lymphoid tissue and a transplantable lymphosarcoma. Incubations were conducted in Krebs-Ringer-phosphate buffer medium. Solid lines represent control animals; dotted lines, adrenalectomized; dashed lines, hormone-injected. Each point is the average of 6 to 12 determinations.

Table 1. The influence of the adrenal cortex on aerobic glycolysis by normal rat lymphoid tissue and a transplantable lymphosarcoma*

Tissue	Treatment	Number of rats	Glucose utilization rate†	Lactate accumulation† (as lactic acid)
Mesenteric Lymph Nodes	Control	13	1.76 ±0.12	0.80 ±0.11
	ACTH or ACE	9	1.78 ±0.13	0.72 ±0.08
	Adrx	10	1.59 ±0.09	0.56 ±0.10
Lymphosarcoma	Control	9	2.03 ±0.15	1.50 ±0.20
	ACTH or ACE	10	4.22 ±0.20	1.63 ±0.15
	Adrx	7	1.97 ±0.12	1.39 ±0.13

* All incubations were carried out for 2 hours in a rat serum medium at 38° C. with a constant stream of 95 per cent oxygen, 5 per cent carbon dioxide passing through the flask.

† The figures shown are expressed as mg. per hour per gm. wet wt. tissue (average ± standard error).

tissue. On the other hand, adrenal cortical hormones stimulated the glucose utilization rate of the tumor tissue under these conditions. The accumulation of lactic acid during the incubation of normal mesenteric lymph nodes and lymphosarcoma tissue in serum is indicated in Table 1. Lactate accumulation was considerably higher in the tumor tissue incubates. The adrenal cortical secretions produced no striking effect on lactate accumulation in either normal or malignant lymphoid tissue incubates.

DISCUSSION

The profound histological changes which occur in normal lymphoid structures as a result of pituitary-adrenal cortical hyperactivity (Dougherty and White 1945) were not accompanied in the present investigations by striking alterations in the determined metabolic activities of normal rat lymphoid tissues *in vitro*. Previous adrenalectomy, it is true, tended to depress lymphoid tissue metabolism. This depression, however, cannot certainly be ascribed to a lack of circulating adrenal cortical hormones in view of the absence of an accentuating effect of hormone-treatment.

Perhaps of more interest were the observations made of the influence of the adrenal cortical secretions on the aerobic metabolism of malignant lymphoid tissue. The addition of glucose to tumor incubates significantly depressed the respiration of this tissue, but had little or no effect on the respiration of normal lymphoid tissue metabolism. The obvious inference was that glucose supplementation of the medium permitted the tumor to obtain more of its energy from glycolysis. This was borne out by the observation that lymphosarcoma tissue converted the major proportion of added glucose to lactate even under aerobic conditions.

The dependence of tumors of all kinds on glycolytic mechanisms for energy has long been recognized (cf. Warburg, 1930). In the present studies, this dependence may well have been related to the stimulating effect of the adrenal cortical secretions on the aerobic utilization of glucose by the tumor. Thus, the previous administration of ACTH or ACE was observed to accelerate the disappearance of glucose in the presence of surviving lymphosarcoma tissue. However, a concomitant increase in lactate accumulation or oxygen consumption did *not* occur. It seems likely, therefore, that the accentuated utilization of carbohydrate resulting from hormone administration was accompanied by the sparing of endogenous substrate.

The relationship of the above observations to the growth-arresting effect of cortisone and ACTH on lymphoid tumors is at present obscure. It is possible that an increased requirement for carbohydrate under these conditions may be the limiting factor responsible for tumor regression.

SUMMARY

Adrenalectomy, ACTH, and adrenal cortical extract (ACE) were without striking effect on the respiration or aerobic glycolytic activity of surviving normal rat lymphoid tissue, including spleen, thymus, and mesenteric lymph nodes.

In contrast, the aerobic utilization of glucose by lymphosarcoma tissue *in vitro* was found to be significantly elevated by previous treatment of the animal supplying the tissue with ACTH or ACE. This increase was not accompanied by a change in lactate accumulation or total oxidative metabolism. The possible relationship of these findings to the growth-arresting effect of the adrenal cortical secretions on lymphoid tumors is discussed.

Acknowledgment

The technical assistance of Miss Irene Rask is hereby gratefully acknowledged.

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EFFECTS OF AMPHENONE "B" ON THYROID AND ADRENAL FUNCTIONS[1]

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AMPHENONE "B"[3] (Allen and Corwin, 1951), a substituted desoxybenzoin, given orally or subcutaneously, produces marked enlargement of rat thyroid and adrenal glands (Hertz, Allen and Tullner, 1950; Hertz, Tullner and Allen, 1951; Hogness, Williams and Lance, 1952). The thyroid enlargement is associated with reduction in I^{131} concentration (Hogness, Williams and Lance, 1952) and a lowered metabolic rate (Holtkamp, Heming and Sax, 1952). The thyromegalic effects are not found in hypophysectomized rats (Hertz, Allen and Tullner, 1950) or in those given supplementary thyroid extract (Hertz, Tullner and Allen, 1951). The adrenomegaly produced by Amphenone "B" is associated with increase in cholesterol concentration (Hogness *et al.*, 1952) without evidence of decreased adrenal function (Hertz *et al.*, 1951; Hogness *et al.*, 1952; Heming *et al.*, 1952). As in the case of the thyroid, adrenal enlargement is not found in hypophysectomized animals (Hertz *et al.*, 1950) or in those given cortisone (Hertz *et al.*, 1951).

In the course of previous studies with this compound certain questions arose. A marked reduction of radioiodine concentration in the thyroid occurred following the injection of Amphenone "B." Although this probably represented inhibition of I^{131} uptake, the possibility of an increased rate of release of iodine from the gland existed. In the case of the adrenal, most evidence indicated normal function following Amphenone "B" administration. However, the possibility of a partial block of steroid output remained (Hertz, Tullner and Allen, 1951). Because of possible clinical uses of a compound with such marked effects upon both thyroid and adrenal function, it seemed important to investigate these problems, and it

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[3] Kindly supplied by Dr. Arthur E. Heming, Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

was with this purpose in mind that the following experiments were designed.

METHODS

Sprague-Dawley rats were used in all experiments; age, weight and sex varied with the experiment but were the same within any given group and its controls. The animals were fed Purina laboratory chow and were kept at constant temperature of 78° F. Amphenone "B" was administered by subcutaneous injection, unless otherwise noted.

Thyroid: In order to determine more definitely whether Amphenone "B" exerted an inhibitory or secretory effect, the following experiments were conducted. First, thyroid glands were removed from a series of male rats weighing approximately 200 grams and placed in Krebs-Ringer solution, pH 7.4, containing Amphenone "B" in molar concentrations ranging from 10^{-3} through 10^{-7}. The thyroids from four animals were used at each concentration. Immediately upon introduction of the glands into the solutions, 10 μc. of I^{131} was added to each flask. The flasks were incubated in a Dubnoff shaker at 37° for one hour, whereupon the glands were removed, washed, placed in 6N NaOH, homogenized, and radioactivity assayed by means of a Geiger-Mueller counting system. Results were expressed as the per cent of I^{131} uptake exhibited by the control glands. For purposes of comparison an identical experiment was carried out, substituting propylthiouracil for Amphenone "B."

In a second experiment four 100 gm. male rats were given 20 mg. of Amphenone "B" in 0.5 ml. of normal saline, intravenously. After one hour, these rats and four controls were given 25 μc. of I^{131} in saline subcutaneously. Four hours later the rats were sacrificed and radioactivity in each lobe of the thyroid determined. Results were expressed as per cent of injected I^{131} per mg. of thyroid tissue.

The following experiment was designed to determine whether Amphenone "B" caused evacuation of radioiodine from the thyroid. Fifteen male rats weighing 200 to 250 gm. were given 25 μc. of I^{131} subcutaneously. Five hours later the animals were sacrificed, the thyroid glands removed and the two lobes separated. One lobe from each animal was incubated in Krebs-Ringer solution containing 10^{-3} M Amphenone "B," while the other lobe was incubated in Krebs-Ringer solution containing 10^{-3} M propylthiouracil; all flasks contained 0.001% KI in order to block I^{131} reincorporation. After incubation for 90 minutes in a Dubnoff shaker, the glands were placed in 6N NaOH, homogenized and radioactivity assayed.

Another experiment similar to the above was conducted; however, in this case one half of the lobes were incubated in Krebs-Ringer solution containing KI and propylthiouracil and the other half were incubated in Krebs-Ringer solution containing KI alone, as a control.

Adrenal: To establish more conclusively (a) whether Amphenone "B"-induced adrenomegaly is associated with adrenal blockade or a normal or increased output of adrenal steroids and (b) whether Amphenone "B" exerts an immediate effect on the adrenal, similar to ACTH, the following two experiments were devised.

Eighteen 90 gm. female rats were given Amphenone "B" twice daily for four and one-half days. The dosage was 10 mg./100 gm. body weight and the drug was administered subcutaneously in 0.5 ml. of physiological saline, pH 4.0. Twelve hours before sacrifice all animals were given the last injection of Amphenone "B" and food was withdrawn. Four hours before death six of the animals were given a subcutaneous injection of 1 I.U. of ACTH,[4] six were placed in the cold (for 4 hours) and six served as controls. At the was 26 mg./100 gm. body weight. This difference is significant ($P < 0.01$). Neither ACTH nor exposure to cold altered the adrenal weight.

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[4] Kindly supplied by Dr. Grosvenor Bissell, Armour and Co., Chicago, Illinois.

The center graph in Figure 4 represents the adrenal ascorbic acid content. Whereas there appears to be some increase in ascorbic acid concentration in the Amphenone "B" treated animals as compared with the controls (mean values 0.446 and 0.400 mg./100 mg. of adrenal respectively) this difference is not statistically significant. However, within each group the differences in adrenal ascorbic acid concentration following ACTH or cold stimulation were highly significant. Injection of 1 I.U. of ACTH into Amphenone "B" treated rats resulted in a fall in adrenal ascorbic acid

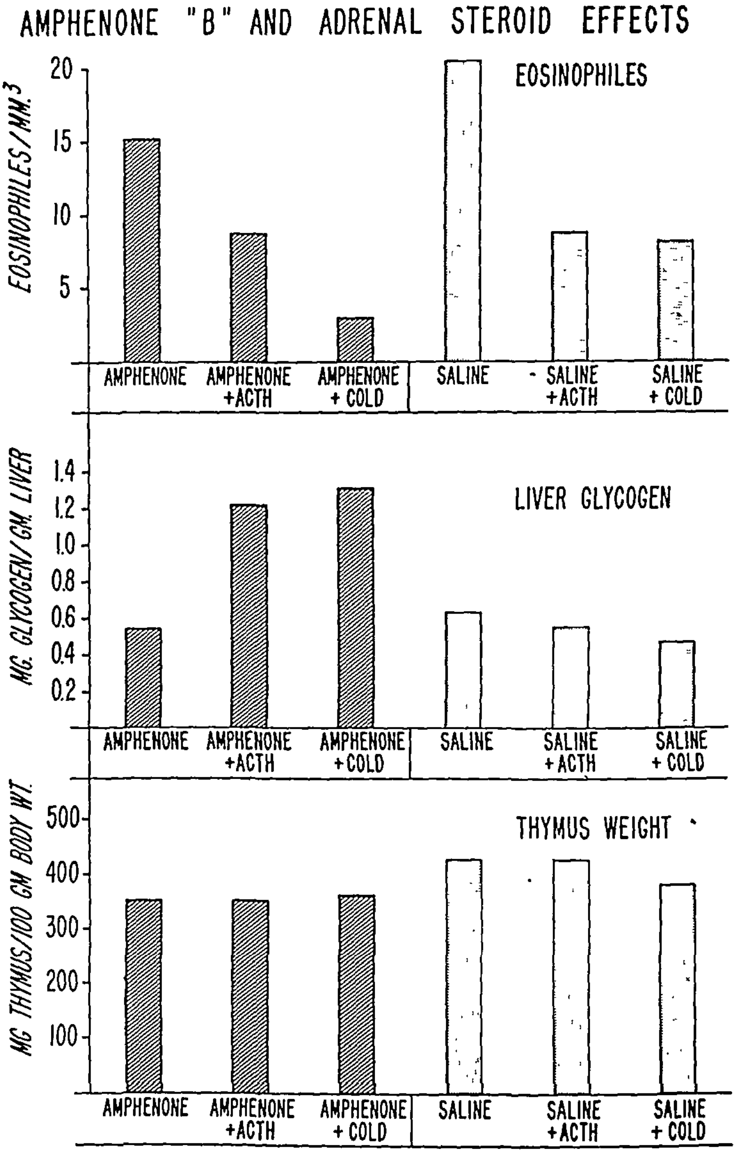

FIG. 5

from a mean of 0.446 mg./100 mg. of adrenal to 0.356 mg. ($P < 0.001$) and subjection to cold for four hours produced a more marked decrease to a mean of 0.277 mg./100 mg. of adrenal. In the saline controls, under conditions of both ACTH and cold stimulation, the ascorbic acid concentration fell from a mean of 0.400 to 0.344 and 0.345 respectively ($P < 0.03$). These observations are of interest in that in Amphenone "B" treated animals, ACTH or cold stimulation produced a greater adrenal response than was seen in the saline controls. It would also appear that four hours in the cold was a greater stimulus than subcutaneous administration of 1 I.U. of ACTH.

The adrenal cholesterol concentration seen in the lower graph in Figure 4 paralleled the observations on ascorbic acid. A more than two fold increase in cholesterol concentration was observed in the Amphenone "B" treated animals, confirming previous observations. However, despite this increase, cold stimulus for example, produced a marked fall in cholesterol concentration from a mean of 6.86 mg./100 mg. adrenal to a mean of 5.02 mg. ($P < 0.01$). As may be seen from the graph, this response was of greater magnitude than that seen in the controls. As was the case with the ascorbic acid determinations, cold appeared to produce a greater stimulus than the injection of one I.U. of ACTH.

Figure 4 represents direct measurements of adrenal properties. However, if a stimulus to the adrenal is to be effective it must produce not only changes in the adrenal itself but also an increase in production and secretion of adrenal steroids. Such an increased output might be reflected by changes in (a) circulating eosinophiles (b) liver glycogen content and (c) thymic weight. Figure 5 shows that such changes do occur under the conditions of this experiment.

Following ACTH or cold stimulation there was a significant fall in circulating eosinophiles in both the Amphenone "B" treated animals and the controls. Again, this fall is more pronounced with cold exposure, especially in the case of the Amphenone "B" animals. Although the absolute eosinophile determinations seem somewhat higher in the controls than in the Amphenone "B" animals (20.4 $mm.^3$ as compared with 15.2 $mm.^3$) this difference is not of significance statistically.

Although the fasting liver glycogen is the same in Amphenone "B" treated animals and saline controls, a highly significant increase in liver glycogen occurred when the Amphenone "B" animals were injected with 1 unit of ACTH or subjected to four hours in the cold. A rise of from 0.54 mg. of glycogen per gram of liver to 1.22 and 1.31 mg. respectively, resulted ($P < 0.02$). No such rise occurred in the control group, indicating again that the Amphenone "B" stimulated gland is capable of an even more marked response to ACTH and cold than the controls.

The bottom graph in Figure 5 represents thymic weights in Amphenone "B" and control groups. The mean thymus weight in the control rats was 425 mg./100 gm. body weight, whereas the mean in the Amphenone "B" treated rats was 351 mg. This difference is significant ($P < 0.01$). No significant differences were found within Amphenone "B" and control groups, however.

The results of an acute experiment, in which one injection of Ampehenone "B" was given four hours before death, are shown in Table 2. No differences of any significance were observed between the values obtained in the animals given no injections, those injected with saline, and those to whom Amphenone "B" was administered. This indidates that, with respect to the adrenal, one injection of Amphenone "B" does not exert a measurable immediate effect.

TABLE 2. EFFECT OF SINGLE SUBCUTANEOUS INJECTION OF AMPHENONE "B" ON ADRENALS

	Control	Saline	Amphenone "B"
Adrenal weight (mg./100 gm. body wt.)	30.2	29.4	30.5
Ascorbic acid (mg./100 mg. adrenal)	0.37	0.42	0.31
Cholesterol (mg./100 mg. adrenal)	2.15	2.04	1.87
Thymus weight (mg./100 gm. body wt.)	161	154	169
Eosinophiles (per mm.^{3})	38.3	34.0	39.2
Liver glycogen (mg./gm. liver)	3.3	3.1	2.3

DISCUSSION

It has been shown that prolonged administration of Amphenone "B" results in thyroid enlargement. The studies reported in this paper show than Amphenone "B," in low concentration, inhibits the incorporation of radioactive iodine into the thyroid both *in vitro* and *in vivo*. On a molar basis Amphenone "B" was shown to be as effective a thyroid blocking agent as is propylthiouracil. Whereas both agents produced marked inhibition of thyroid I^{131} uptake, neither produced any change in the rate of release of iodine.

It is concluded that the increase in thyroid size observed following Amphenone "B" administration occurs on the same basis as with propylthiouracil. It is presumably the result of an increase in circulating TSH brought about by a fall in the blood thyroxine level, resulting from antagonism to its synthesis. This conclusion is supported by the observation of Holtkamp, Heming and Sax (1952) of a lowered oxygen consumption following Amphenone "B" administration.

The observations on the thyroid suggested that the adrenal enlargement and associated rise in cholesterol concentration noted after prolonged Amphenone "B" administration might also be the result of a "blocking" action. Contrary to original expectations however, no diminution in adrenal function was observed. Evidence presented indicates that the adrenals of the Amphenone "B" treated animal are capable of responding to stimulation by secreting an even greater quantity of steroids than those of the controls. In animals receiving Amphenone "B" over a four and one-half day period the administration of 1 I.U. of ACTH or exposure to cold for four hours produced a marked fall in adrenal ascorbic acid and cholesterol concentrations. This was associated with a pronounced rise in liver glycogen and a significant fall in circulating eosinophiles. These changes were more marked in the Amphenone "B" treated animals than in the control group. Therefore, not only are the adrenals of the Amphenone "B" treated animals capable of responding to stimulation but it would seem reasonable to conclude that they respond by secreting a greater quantity of steroids than the controls. Of interest was the observation that, in the Amphenone "B" treated animal, exposure to cold for four hours apparently evokes a greater adrenal response than one I.U. of ACTH administered subcutaneously.

The conclusions reached above appear to be quite the opposite of the situation observed in the case of the thyroid. Thiouracil has been shown to produce an effect on the adrenal (Zarrow and Money, 1949) as well as on the thyroid, but in this instance the changes observed consisted of a true cortical atrophy. Hence, although Amphenone "B" acts like thiouracil where the thyroid is concerned, the action on the adrenal is very different from that of thiouracil.

In a previous experiment (Hogness, Williams and Lance, 1952) it was shown that administration of 35 mg. of Amphenone "B" in four divided doses, over a 24-hour period, produced no change in adrenal size, adrenal cholesterol concentration or thymus size. Therefore Amphenone "B" does not exert an effect like short term administration of ACTH, formaldehyde, or other compounds that produce an acute "alarm" response. The reaction is also unlike that observed after the administration of such substances as erucic acid where an increase in adrenal size and cholesterol content has been noted but no change in steroid secretion was apparent (Carroll, 1951; Carroll and Noble, 1952).

Since Amphenone "B" does not exert a blocking effect on the adrenal and since the increase in adrenal size is not produced in hypophysectomized animals, our results can be explained by postulating that the compound stimulates the anterior pituitary or hypothalamus directly and that the adrenomegaly is the result of an increase in trophic hormone brought about by this stimulus.

This interesting compound has three distinct sites of action. It produces progestational effects (Allen, Hertz and Tullner, 1950) presumably by a direct action on the end organs, since these changes occur even in hypophysectomized animals (Hertz *et al.*, 1950). It also acts directly on the thyroid by inhibiting iodine uptake and hence suppressing thyroid function, and finally, the compound apparently has a direct stimulatory action on the pituitary, producing adrenomegaly in this manner.

SUMMARY

1) Amphenone "B" was shown to inhibit the incorporation of I^{131} into thyroid protein both *in vivo* and *in vitro*. This effect was present when molar concentrations of the inhibitor were as low as 10^{-6}, and the compound was shown to be equally as potent an anti-thyroid substance *in vitro* as propylthiouracil. Neither Amphenone "B" nor propylthiouracil was shown to have any effect on the rate of release of radioiodine from the rat thyroid.

2) Prolonged administration of Amphenone "B" to rats produced adrenomegaly which was associated with increases in adrenal ascorbic acid and cholesterol concentrations. Determinations of liver glycogen, circulating eosinophiles and thymus weight indicated that the enlarged adrenals were capable of responding to ACTH or cold stimulus by secreting an increased quantity of steroids. The response in Amphenone "B" treated animals was even greater than that of the controls.

3) It is concluded that Amphenone "B" has a direct antithyroid "blocking" action but that the effect on the adrenal is stimulatory in nature.

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THE PROTEIN ANABOLIC EFFECT OF TESTOSTERONE PROPIONATE AND ITS RELATIONSHIP TO INSULIN

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SINCE Kochakian and Murlin (1935, 1936, 1937) performed their fundamental experiments on castrate dogs it has been well established that administration of androgenic substances causes a fall in the non-protein nitrogen level (NPN) in blood and at the same time lowers the nitrogen excretion in the urine. It is well known also that insulin has a very similar effect when injected into the diabetic organism (Falkenhausen, 1925, Chaikoff and Forker, 1950)—in other words, insulin and the male sex hormone may, under certain circumstances, exert a similar protein anabolic effect. The interrelationship between testosterone propionate and insulin has therefore been investigated and as a first step the behavior of the NPN and blood sugar levels of normal and depancreatized dogs receiving testosterone has been studied.

EXPERIMENT

This study was carried out on eight well trained female dogs of a weight between 20–25 Kg. Three of them were surgically depancreatized and a standard diet was fed twice a day. Regular (Toronto) insulin was used and administered subcutaneously twice a day in order to keep the urine of the animals sugar-free. Studies were made on 1) normal dogs, 2) totally depancreatized dogs after withdrawal of insulin and on 3) depancreatized dogs receiving various doses of insulin. Each experiment was carried out twice on the same dog, once without testosterone and again after injection of testosterone.

The injections of the male sex hormone (we are grateful to the Ciba Company for supplies of Perandren) were given in the following way: the first morning 50 mg. of testosterone propionate dissolved in Sesame oil were given intramuscularly, the second day 50 mg. in the morning and 25 mg. in the afternoon. The third day the actual experiment was carried out when 4–7 blood samples were drawn from the animal which was fasting for 24 hours before start of the experiment. A dog treated with testosterone was used again only after an interval of at least 4–5 weeks when it was assumed that all injected testosterone had disappeared from the body.

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Blood sugar was determined according to Miller and Van Slyke (1936) and for NPN determinations on whole blood Folin and Wu's (Boström, 1944) colorimetric method was applied. The precipitation of blood proteins was carried out according to a special modification described by Folin (1930). In this not very common form of protein precipitation blood cells are minimally damaged and haemolysis is prevented. Using this modified precipitation method the results are around 12 mg.% lower than figures obtained by the original method of Folin and Wu and even small variations in the NPN level can be detected.

RESULTS

The dose of 125 mg of testosterone propionate injected into a normal dog under the standardized conditions of our experiments proved to be highly effective as demonstrated in Figure 1. A similar depression of the NPN level in blood was obtained also in the other four intact animals. In contrast to normal dogs, the same dose of testosterone injected into our totally depancreatized dogs did not exert any effect on the NPN level when the exogenous supply of insulin was withheld for 24 hours before the start of experiment. Figures 2a and 2b are good illustrations of the negative response of diabetic dogs when only traces of insulin were present in the body. When under the same conditions an arbitrarily chosen dose of insulin was injected into such a diabetic dog a remarkable lowering of the

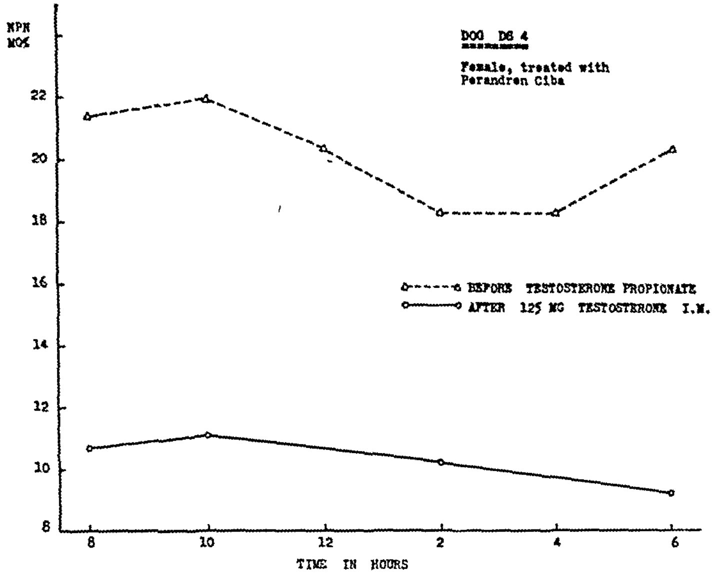

FIG. 1. The normal female dog responds to testosterone by a remarkable lowering of the non-protein nitrogen level in blood.

NPN occurred, as shown in Figure 3, and no further depression of the NPN level could be obtained when the same dose of insulin and testosterone were acting together at the same time. The same observation was made after injections of even smaller amounts of insulin, such as 7, 4 or 2

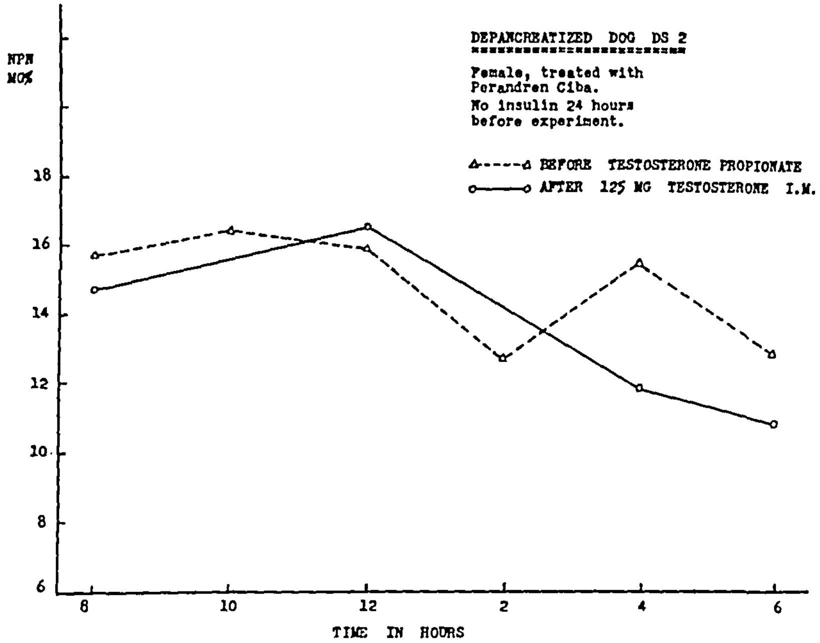

FIG. 2a. The depancreatized dog kept for 24 hours without exogenous insulin exhibits no change of the non-protein nitrogen level after testosterone treatment. Dog DS2.

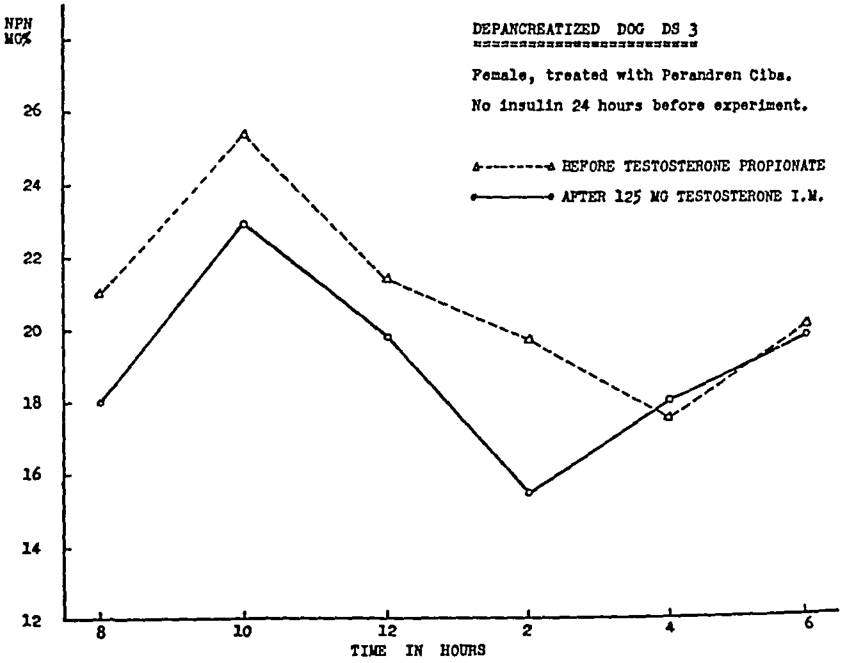

FIG. 2b. Same as 2a. Dog DS3.

units. The fall of the NPN, however, as could be expected *a priori*, after smaller doses of insulin was not so dramatic as shown in Figure 3, but testosterone never influenced the NPN level in either direction in these depancreatized dogs.

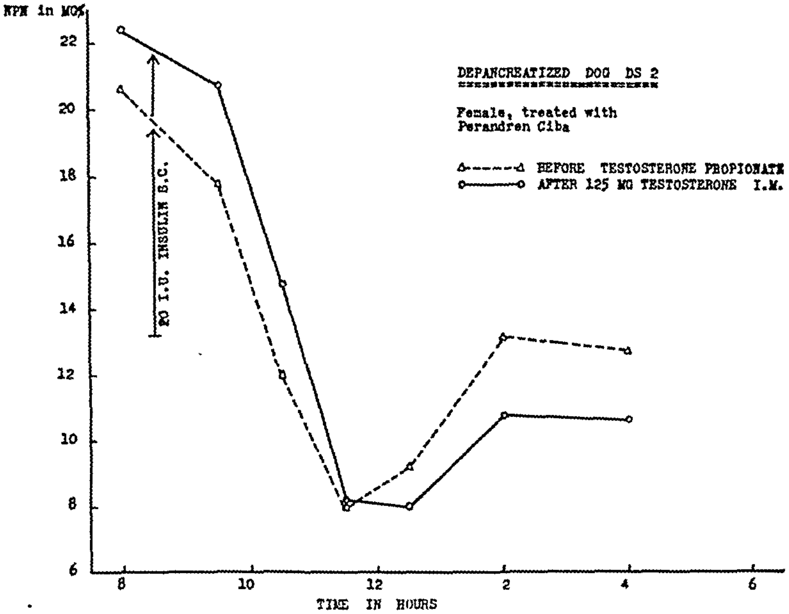

FIG. 3. The diabetic dog responds to insulin by a prompt lowering of the non-protein nitrogen level. Testosterone neither supplements nor counteracts the NPN-lowering effect of insulin.

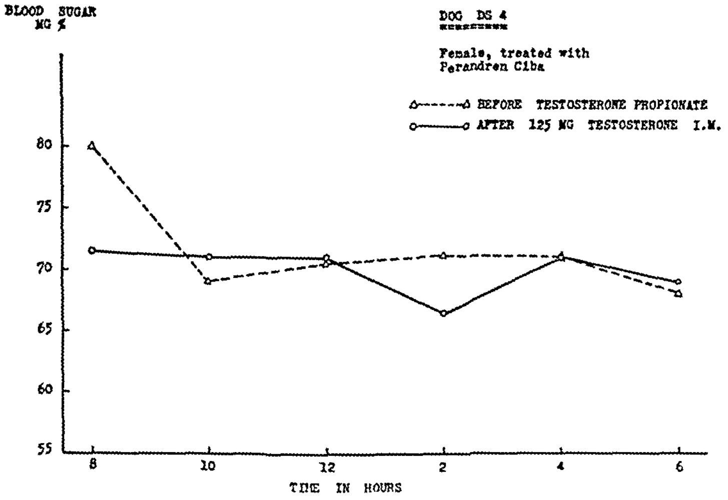

FIG. 4. A standard dose of 125 mg. of testosterone propionate does not affect the blood sugar level of the normal female dog.

CONJOINT EFFECTS OF PYRIDOXINE DEFICIENCY AND ANTERIOR PITUITARY GROWTH HORMONE ADMINISTRATION IN THE RAT

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IT HAS been shown in enzymatic studies (Cammarata and Cohen, 1950; Armstrong, Feldott and Lardy, 1950) and investigations with intact rats (Sheppard and McHenry, 1946; Hawkins, MacFarland and McHenry 1946; Beaton, Ballantyne, Lau, Steckley and McHenry, 1950) that vitamin B_6 affects nitrogen metabolism. Anterior pituitary growth hormone has been stated to promote nitrogen retention (Li and Evans, 1947; Russell, 1951). In order to determine whether the hormone would exert its anabolic influence in rats rendered deficient by dietary restriction of vitamin B_6 and by the administration of the vitamin antagonist, desoxypyridoxine, three studies differing somewhat in experimental detail were performed.

EXPERIMENTAL

In all experiments, albino rats of the Wistar strain were housed in individual screen-bottomed cages and fed either a fat-free, 15% casein diet or a 20% corn oil, 20% casein diet as noted. Except for sucrose, the other ingredients in per cent by weight were common to both regimens: salts mixture (Steenbock and Nelson, 1923) 4, vitaminized casein 4 (the protein content included in the total casein), agar 2, choline 0.2, inositol 0.2 and cod liver oil concentrate .015 (supplying approximately 45 I.U. vitamin A and 11 I.U. vitamin D per rat per day). Sucrose made up the remainder of the diet in each case. The vitaminized casein was a mixture of: 100 mg. thiamine chloride, 100 mg. riboflavin, 400 mg. calcium pantothenate, 400 mg. nicotinic acid, 400 mg. p-aminobenzoic acid, 20 mg. biotin, 20 mg. folic acid and 800 gm. casein. All animals were given daily supplements of 100 μg. desoxypyridoxine per rat per day in the diet; control animals were also provided with 50 μg. pyridoxine hydrochloride per rat per day in the diet. The rats receiving anterior pituitary growth hormone[1] were injected with 1 mg. in 1 cc. saline per rat per day in a single dose. Injections of saline of the same volume were given to the remaining rats.

Alanine-load tests were carried out with both vitamin B_6-deprived and

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[1] Armour Laboratories 22KR1.

pyridoxine-supplemented groups by injecting, intraperitoneally, 15.2% DL-alanine in water in quantity to provide 0.5 mg. nitrogen per gm. body weight based on the average body weight per group. One group of animals on each treatment was sacrificed at set intervals of time following the amino-acid administration. Zero hour groups received 5 cc. 0.9% saline per rat by the intraperitoneal route and were sacrificed immediately. Subsequent to anesthetization with nembutal, blood was removed from the exposed heart, heparinized, and pooled for each group. The following analyses were done on the pooled samples: urea (Archibald, 1945), amino nitrogen (Frame, Russel and Wilhelmi, 1943), plasma free glutamic acid and glutamine (Prescott and Waelsch, 1947), sugar (Nelson, 1944), hemoglobin (Collier, 1947), and hematocrit value by the standard method.

In studies involving nitrogen excretion two rats were maintained in each metabolism cage for the last 8 to 11 days of the exeprimental periods. Excreta were collected at 48-hour intervals, toluene being the urinary preservative employed, and analyzed for total nitrogen by a macro-Kjeldahl method. After the removal of certain organs the remaining carcasses were frozen in liquid air, passed through a power grinder, and a representative sample homogenized in a Waring Blendor. Aliquots were taken for determinations of total nitrogen, of moisture by drying in an electric oven at 105° C. for 5 hours, and of total crude fatty acids (Gavin and McHenry, 1940). Homogenized pooled livers were analyzed for water and protein and also for total vitamin B_6 content (Atkin, Schultz, Williams and Frey, 1943).

Experiment I.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pyridoxine-supplemented Adult Female Rats.

Sixteen groups, each of 7 female rats with an average initial body weight of 170 gm., were fed the fat-free, 15% casein diet for 25 days before the growth hormone treatments were begun with 8 groups, the other 8 groups not receiving the hormone. Following 7 days of injections the animals were fasted for 16 hours, doses of alanine administered and the levels of blood metabolites determined at intervals of 0, 1, 3 and 6 hours.

Experiment II.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pyridoxine-supplemented Rats Fed *ad libitum*.

Groups of 8 rats each, of both sexes, with an average initial body weight of 100 gm. were fed the 20% corn oil, 20% casein diet. As a consequence of some mortality those 12 groups which were not used for studies of nitrogen retention were rearranged into 8 groups. After 19 days on diet growth hormone was injected for the next 7 days. Alanine was administered after an 18-hour fast and blood metabolites were measured at intervals of 0, 1½ and 4 hours.

Experiment III.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pair-fed Control Rats.

Four groups of 8 rats each with an average initial body weight of 89 gm. received the same diet and treatments as those of experiment II except that each control group was pair-fed with its correspoding deficient group. Also, no alanine was given in this experiment. The animals were on diet 15 days before the injections of growth hormone were begun and 10 days afterwards.

RESULTS AND DISCUSSION

During the periods that the rats were on experimental diets the greatest differences in the average body weight of groups were observed in experiment II where the rats were smaller than the females used in experiment I and were fed *ad libitum* unlike those of experiment III. In the last experiment the restriction in food intake prevented the typical effect of growth hormone on body weight. This result would be anticipated since an increase in weight can occur only when quantities of nutrients in excess of maintenance requirements are available for storage. Lee and Schaffer (1934) reported that paired-feeding of growth hormone treated rats and uninjected controls does not prevent an increase in body weight consequent to hormone administration. It should be noted that the average food intakes of those animals (about 12 gm. per day) were far in excess of the average food intakes of the animals in experiment III (6 gm. per day). The administration of growth hormone in all experiments increased the severity of acrodynia, the dermatitis characteristic of pyridoxine deficiency.

TABLE 1. AVERAGE BODY WEIGHT OF EXPERIMENTAL ANIMALS

Exp.	Group	Final average body weight	Weight gains during injection period
		gm.	gm.
I	$+B_6$	197	3
	$-B_6$	165	1
	$+B_6+GH$[1]	207	13
	$-B_6+GH$	166	2
II	$+B_6$	179	7
	$-B_6$	109	2
	$+B_6+GH$	189	17
	$-B_6+GH$	102	−9
III	$+B_6$	141	15
	$-B_6$	104	8
	$+B_6+GH$	140	17
	$-B_6+GH$	100	6

[1] GH indicates groups injected with growth hormone.

From the blood analyses it is evident that in the adult female rats, growth hormone had no effect on the typical picture of urea formation from alanine (Beaton *et al.*, 1950) other than to depress the level of blood urea following alanine administration in both control and deficient groups. The levels of the other blood metabolites indicated that the hormone accentuated the effects of the vitamin deficiency. In the studies with the younger rats, experiments II and III, the fasting level of blood urea was elevated in pyridoxine deficiency as was shown previously by Beaton *et al.* (1950). The effect of growth hormone was apparently to raise it further in the deficient and to depress it in the control animals. Changes in the levels

TABLE 2. BLOOD ANALYSES

Exp.	Group	Hours after alanine load	Blood					Plasma	
			Packed cell volume	Hemoglobin	Amino nitrogen	Urea	Sugar	Glutamic acid	Glutamine
			%	gm.%	mg.%	mg.%	mg.%	mg.%	mg.%
I	$+B_6$	0	46	—	12.6	23	183	1.7	13.5
		1	58	—	36.0	43	207	4.3	—
		3	56	—	15.4	80	165	4.1	—
		6	55	—	12.8	116	156	4.2	—
	$-B_6$	0	44	—	12.8	26	141	3.0	12.5
		1	59	—	44.0	40	185	9.0	—
		3	56	—	17.5	83	229	3.3	—
		6	58	—	15.0	121	156	2.8	—
	$+B_6+GH$	0	43	—	14.2	31	141	2.7	11.0
		1	56	—	34.2	38	207	4.2	—
		3	56	—	15.6	69	223	3.0	—
		6	54	—	12.6	94	177	3.3	—
	$-B_6+GH$	0	44	—	14.0	28	177	2.2	9.5
		1	57	—	50.0	35	163	10.7	—
		3	56	—	20.8	62	255	6.3	—
		6	54	—	14.6	101	129	4.6	—
II	$+B_6$	0	51	15.0	10.3	22	75	5.0	5.5
		1½	51	14.7	45.0	44	190	19.4	—
		4	51	13.9	23.5	90	222	8.3	—
	$-B_6$	0	49	14.4	10.5	32	135	4.6	8.5
		1½	49	14.7	46.2	71	88	13.5	—
		4	48	12.7	23.8	130	247	11.0	—
	$+B_6+GH$	0	49	14.6	13.4	19	84	4.4	6.8
		1½	53	14.7	48.6	30	138	10.4	20.2
		4	50	14.7	23.8	88	230	6.3	—
	$-B_6+GH$	0	46	13.3	11.3	36	160	6.9	14.3
		1½	44	12.7	42.3	65	135	14.3	13.3
		4	48	13.9	14.7	136	215	6.9	—
III	$+B_6$	—	43	13.5	12.9	31	145	3.4	5.0
	$-B_6$	—	42	12.7	12.0	44	105	3.6	4.2
	$+B_6+GH$	—	44	12.3	28.2	25	140	2.2	8.0
	$-B_6+GH$	—	45	12.9	13.0	51	160	4.9	9.3

of plasma glutamic acid and glutamine showed no consistency as a consequence of growth hormone administration.

In control animals growth hormone induced nitrogen retention in the rats fed *ad libitum* but not in those with a restricted food intake. Such a finding was in accord with observations on body weight. Although differences in nitrogen retention and body weight were not seen in the pair-fed animals, growth hormone did affect the levels of blood metabolites. In the absence of pyridoxine, the anabolic properties of the hormone were at no time exhibited.

Analyses for moisture, protein and total crude fatty acids of the body showed that administration of growth hormone had no significant effect on the proportions of water, protein, or of fat in either control or deficient rats.

TABLE 3. NITROGEN EXCRETION

Exp.	Group	No. of rats	No. of days injected	Urinary N / Food N	Fecal N / Food N	food N − (Urinary + fecal N)
						gm./rat/day
II	$+B_6$	8	4, 5	.33	.050	.15
		8	6, 7	.49	.050	.11
	$-B_6$	8	4, 5	.47	.042	.09
		8	6, 7	.50	.057	.08
	$+B_6+$GH	8	4, 5	.22	.035	.27
		8	6, 7	.24	.041	.22
	$-B_6+$GH	8	4, 5	.38	.039	.12
		7	6, 7	.59	.084	.05
III	$+B_6$	8	7, 8	.44	.037	.14
		8	9, 10	.62	.036	.07
	$-B_6$	8	7, 8	.74	.065	.05
		7	9, 10	.77	.055	.03
	$+B_6+$GH	8	7, 8	.48	.045	.10
		8	9, 10	.47	.028	.10
	$+B_6+$GH	8	7, 8	.83	.066	.02
		6	9, 10	.69	.057	.06

TABLE 4. LIVER AND CARCASS CONSTITUENTS FOR EXPERIMENT III

Group	Liver		Carcass		
	% water	% protein	% water	% protein	% crude fatty acids
$+B_6$	73.0	19.8	66.1	23.3	9.6
$-B_6$	73.5	18.7	69.2	24.1	4.0
$+B_6+$GH	70.4	20.2	65.6	21.9	9.6
$-B_6+$GH	78.1	19.1	72.0	25.4	2.9

From the assay of total vitamin B_6 in the liver it is seen that its content was lowered by pyridoxine deficiency per se, and again by growth hormone administration in both control and deficient groups, another finding which suggests that the hormone aggravates the state of the deficiency.

In pyridoxine deficiency the absolute and relative weight of the thymus was greatly reduced. Administration of growth hormone appeared to further that effect. The weight of the adrenal glands increased in proportion to the total body weight in the animals lacking pyridoxine. Again in this respect, growth hormone made the effect more pronounced.

TABLE 5. LIVER TOTAL VITAMIN B_6 CONTENTS AND ORGAN WEIGHTS

Exp.	Group	Total vitamin B_6 of liver µg./gm. moist tissue	Liver % body weight	Spleen % body weight	Thymus % body weight	Adrenal gland % body weight
I	$+B_6$	8.3				
	$-B_6$	6.2				
	$+B_6+GH$	8.4				
	$-B_6+GH$	4.7				
II	$+B_6$	7.0	7.7	0.53	0.37	.008
	$-B_6$	5.4	3.4	0.49	0.07	.011
	$+B_6+GH$	6.7	7.8	0.46	0.31	.011
	$-B_6+GH$	4.8	7.7	0.63	0.07	.018
III	$+B_6$	5.7	5.3	0.57	0.31	.014
	$-B_6$	2.9	5.7	0.48	0.11	.015
	$+B_6+GH$	4.7	5.7	0.69	0.24	.016
	$-B_6+GH$	3.0	5.8	0.36	0.07	.022

In studies of vitamin deficiencies the specificity of effects is always open to question. In most deficiency states there is a decrease in food consumption which gives rise to inanition. The use of pair feeding makes possible comparison between two groups with one variable: presence or absence of a particular vitamin. Observations made on this basis have been reported and discussed above. It should be made clear, however, that the deficiency of only one vitamin has been studied. This one was selected because of the reported relation to protein metabolism. A deficiency of another B vitamin might have given similar results. Until further investigations are made it will not be possible to state that the reported effects are specific for a single vitamin.

SUMMARY

1. Experiments have been described in which pyridoxine deficiency and anterior pituitary growth hormone administration were conjointly studied in adult female rats and in younger rats fed *ad libitum* and on a restricted food intake.

2. The only observed effects of growth hormone on the blood chemistry of pyridoxine-deficient animals following a test-load of alanine were apparent in adult female rats.

3. Provided that the animals were receiving an adequate amount of food, growth hormone promoted increased body weight and nitrogen retention in the control animals but not in the deficient ones.

4. The hormone aggravated the effects of deficiency as demonstrated by the increased severity of acrodynia, the further elevation in the fasting level of blood urea, the lowered content of total vitamin B_6 in the liver, the decreased weight of the thymus, and the increased weight of the adrenal glands. These effects of growth hormone administration on organ weights and on total vitamin B_6 content were also observed in animals provided with pyridoxine.

Acknowledgments

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THE EFFECT OF ASPIRIN UPON THE GLYCOSURIA OF PARTIALLY DEPANCREATIZED RATS IN THE PRESENCE AND ABSENCE OF THE ADRENAL GLANDS

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SALICYLATES and related compounds are capable of activating the adrenal cortices via the anterior pituitary (Cronheim, King and Hyder, 1952). Hailman (1952) has summarized the evidence that the therapeutic and biologic effects of these compounds are mediated by the increased secretory activity of the adrenal cortices.

We have considered the possibility that aspirin can cause a state of hypercorticalism in the laboratory rat. If this were true, it would be expected that aspirin would exacerbate the diabetes of the partially depancreatized rat since corticotropin and the 11-oxygenated steroids of the adrenal cortex are diabetogenic (Ingle, 1948). On the contrary, large doses of aspirin suppress the glycosuria and hyperglycemia of diabetic patients and animals (see Gross and Greenberg, 1948, for review).

In the present study it is shown that large doses of aspirin ameliorate the diabetes of partially depancreatized rats in both the presence and absence of the adrenal glands.

METHODS

Infection-free male rats of the Sprague-Dawley strain were partially depancreatized at a weight of approximately 275 gms. by the procedure described by Ingle and Griffith (1942). The experiments were conducted in an air-conditioned laboratory with the temperature maintained at 74 to 78° F. and the humidity at approximately 50 per cent of saturation. After the animals had recovered from the operation and had reached a weight of approximately 300 gms., they were placed in metabolism cages and were adapted to the force-feeding of a medium carbohydrate diet (Table 1) by stomach tube each morning and late afternoon. The technique and diet were modified from those described by Reinecke, Ball and Samuels (1939). During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of food shock. The animals were brought to a full feeding of 26 cc. of diet per day on the 6th day. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with toluene. Urine glucose was determined by the ferricyanide electrode method of Shaffer and Williams (1935).

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TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	grams
Cellu flour (Chicago Dietetic Supply House)	60
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc.

After the level of glycosuria had become stable, 50 per cent of the animals were adrenalectomized by sterile technique. The amount of adrenal cortical extract required to restore the glycosuria to its preadrenalectomy level (3 to 5 cc. in divided doses per day) was established for each adrenalectomized rat and was then kept uniform throughout the remainder of the experiment. The adrenal cortical extract (Upjohn) was prepared from mixed beef and hog glands and represents the activity equivalent of 0.1 mg. of hydrocortisone per cc.

Aspirin was ground into a fine suspension in peanut oil at a concentration of 100 mg. per cc. It was administered by subcutaneous injection immediately after the morning and late afternoon feedings.

EXPERIMENTS AND RESULTS

Following control periods of several weeks, each rat was treated with 40 mg. of aspirin daily for 7 days, 80 mg. daily for 7 days and 160 mg. daily for 7 days. When the injections were stopped each animal was observed during a second control period of 14 days. The experiments involved 10 severely diabetic rats, 5 of which were adrenalectomized; 13 moderately diabetic rats, 6 of which were adrenalectomized; and 10 mildly diabetic rats, 5 of which were adrenalectomized.

During the administration of aspirin there was a suppression of the glycosuria in each animal to an extent which was proportional to the dose. When the injections of aspirin were stopped, there was exacerbation of the glycosuria to levels much higher than the initial control values. The pattern and extent of response was similar in the adrenalectomized and non-adrenalectomized animals. The data are summarized in Figure 1.

DISCUSSION

The effect of aspirin upon glycosuria in the rat occurs independently of any change in adrenal function. Smith (1952) has shown that sodium salicylate reduces the glycosuria of steroid (cortisone) diabetes in the rat and also suppresses the glycogenic activity of cortisone.

Other biologic effects of salicylates occur independently of the adrenal cortices. Thorn (1951) has noted the usefulness of aspirin in controlling the fever of patients having adrenal cortical insufficiency.

It would be of special interest to know whether or not the therapeutic effect of aspirin and similar compounds upon the rheumatoid diseases is mediated by the adrenal cortices. This hypothesis has found favor among a number of laboratory and clinical investigators but the supporting evidence is indirect and can be otherwise interpreted. A critical trial of the hypothesis would require the testing of these compounds in patients without functional adrenal glands who also have one of the rheumatoid diseases.

SUMMARY

This study was a partial test of the hypothesis that large doses of aspirin can cause a state of hypercorticalism in the rat. Thirty-three partially depancreatized male rats of about 300 gms. weight were force-fed a medium

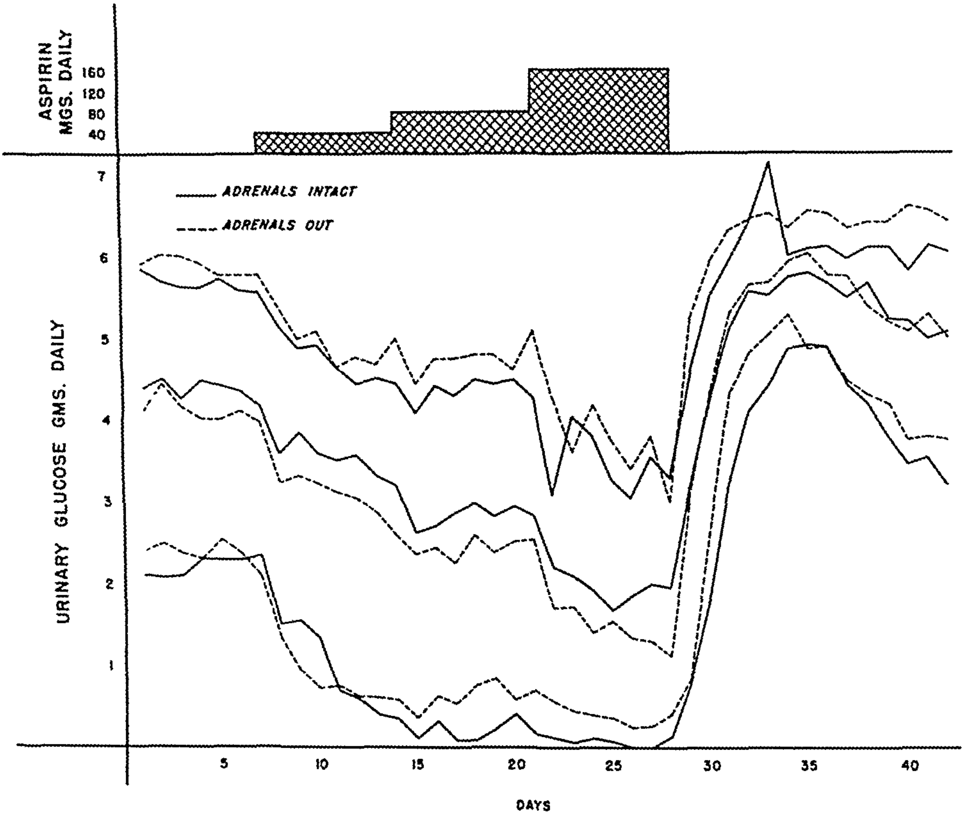

FIG. 1. The effect of aspirin upon the glycosuria of partially depancreatized rats, with and without adrenal glands. Averages.

carbohydrate diet. Sixteen rats of this group were also adrenalectomized and treated with adrenal cortical extract in amounts (3 to 5 cc. daily) which sustained the preadrenalectomy level of glycosuria. Each rat was given subcutaneous injections of aspirin in oil suspension in amounts of 40, 80 and 160 mg. per rat for 7 days per dose. There was a marked suppression of glycosuria to an extent which was related to the size of the dose of aspirin. When the injections of the drug were stopped there was a temporary exacerbation of the glycosuria to much higher levels than were noted during the pre-injection period. Since the pattern and extent of response to aspirin was identical in both the presence and absence of the adrenal glands, it was concluded that this pharmacologic effect of aspirin is not mediated by the adrenal glands.

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ANTAGONISTS TO CORTISONE: AN ACTH-LIKE ACTION OF STEROIDS

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THE experiments reported here were undertaken to determine if some of the effects of cortisone in rats could be antagonized or otherwise modified by the simultaneous administration of other steroids. In the course of the initial studies it was observed that both 17α-methyl-5-androstene-3β,17β-diol, subsequently referred to as MAD, and testosterone propionate (TP) counteracted markedly the adrenal-inhibiting effects of cortisone (Gaunt, Howell and Antonchak, 1952) and subsequent work has been focused largely on that point. By coincidence Winter and Hollings (1951) and Winter, Hollings and Stebbins (1953) independently but earlier observed the same adrenal-maintaining effect of the same two compounds. They put their data at our disposal prior to its publication. We have extended their work particularly by studying the effects of a large number of steroids but in all instances in which duplication occurred we have confirmed their findings.

It has been known since the original work of Cutuly, Cutuly and McCullagh (1938), confirmed by Leonard (1944) and Zizine, Simpson and Evans (1950), that testosterone propionate and some other steroids (Leathem and Brent, 1943; Leonard, 1944) would prevent partially the expected adrenal atrophy after hypophysectomy. Leathem (1944) saw the same effect of testosterone propionate but found that it was a time-limited response which faded out despite continued treatment. We have found that cortisone-induced adrenal atrophy in intact animals can be prevented by testosterone and a large number of other C-19 and C-20, but not C-21, steroids and that this effect cannot be correlated with their androgenic, growth-stimulating or other observed biological properties.

METHODS

Male rats from our inbred Wistar stock weighing approximately 60 grams were used. They were maintained on a commercial laboratory chow supplemented by lettuce and cod liver oil.

Cortisone acetate was given in a dose of 1 mg. per day and other steroids were used in varying dosages. All injections were made subcutaneously. In the initial experiments

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treatments were continued for 20 days. Later it was observed that essentially the same effects could be observed after only seven days of treatment and subsequent experiments were of that duration. Fresh wet weight of organs are recorded.

Since data were too voluminous for all of them to be included in this paper relative organ weights only are tabulated except in Table 5 and 6. Actual weights can easily be estimated from the body weight data. The statistical value included is the standard error $=\sqrt{\sum d^2/n(n-1)}$. Statements that differences between means are definitely significant are based upon p values of less than 0.05 from Fisher's table of t.

Seminal vesicle weights were included as a rough index of the androgenicity of the compounds studied. Since non-castrated animals were used, these figures should not be construed as precise measures of androgenicity.

Cortisone acetate, referred to generally in this paper as cortisone, was given in aqueous suspension. DCA, estradiol benzoate and the 0.25 mg. doses of TP (Tables 1, 2 and 3) were given in oil solution. MAD was administered in an emulsion in the cases recorded in Tables 1, 2 and 3. All the compounds shown in Tables 4 and 6 were injected as microcrystalline aqueous suspensions. No differences attributable to the vehicle were observed.

The adrenal glands were sectioned and examined particularly for changes in lipid content and distribution. One adrenal was fixed in 10 per cent neutral formalin for 48 hours, embedded in gelatin (Zwemer, 1933) and returned to formalin until sectioned. These glands were sectioned at 15 microns on a freezing microtome, stained with sudan IV (Herxheimer's solution, Cowdry, 1943) or with sudan black B and mounted in glychogel (Zwemer, 1933). Some sections were placed in 2.5 per cent ferric ammonium sulfate at 37° C. for three days and then treated with sulfuric acid (Schultz, 1924) in an attempt to reveal cholesterol and its esters. The other adrenal was either fixed in Bouin's fluid or in cold 80 per cent alcohol. The glands fixed in Bouin's were serially sectioned and stained with hematoxylin and eosin. The glands fixed in alcohol were used to estimate the alkaline phosphatase enzyme by the Gomori (1939, 1941) technique with the addition of 2 per cent magnesium sulfate to augment the stain. Such tissues were incubated at 37° C. with sodium beta glycerophosphate for three hours at pH 9.3–9.4.

RESULTS

Adrenal Maintenance

As noted in all of the tables, cortisone when given alone produced the expected adrenal inhibition. This effect was almost as great after 7 as after 20 days of treatment. In hypophysectomized rats cortisone was without effect on the actual adrenal weights but, because of body weight loss, increased the relative adrenal weights.

DCA also reduced adrenal weight (Table 1) but less so than cortisone. When DCA and cortisone were combined their effects in reducing adrenal weight were greater than that of either compound alone in the 20-day experiments (Table 1) but not in those run for seven days (Table 3).

A sodium chloride drinking solution was without effect on adrenal weight when given alone or with cortisone (Table 1). When given with DCA it increased adrenal weight over that seen with DCA alone (Table 1). This may be a manifestation of the expected damaging effects of this combination.

TABLE 1. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT AND ADRENALECTOMIZED MALE RATS[1]

Experiments of 20 days duration

Group No. & treatment	Body weight—gm.			Organ weight (per 100 gm. body weight)					No. rats
	Initial	Terminal	% Gain	Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
Intact Rats									
1. None	65	157	144± 6.0	16.6±0.50	313±15.3	0.91±0.016	1.14±0.023	45.3±2.9	12
2. Cortisone Acetate 1 mg./day	68	121	77± 6.4	7.7±0.55	128±11.9	1.03±0.030	1.42±0.063	53.0±7.8	12
3. DCA 1 mg./day	65	172	165± 9.2	12.9±0.42	304±10.8	1.03±0.013	1.08±0.030	49.0±3.15	12
4. 1% NaCl to drink	63	148	133±10.4	18.2±0.93	299±10.9	0.99±0.010	1.15±0.045	39.5±3.65	12
5. Cortisone Acetate + DCA	64	131	104± 7.7	5.9±0.25	130±21.8	1.19±0.023	1.25±0.051	43.4±3.74	12
6. DCA+NaCl	66	147	123± 9.1	15.3±0.92	270±22.2	1.23±0.035	1.14±0.041	39.4±3.88	12
7. Cortisone Acetate +NaCl	67	109	63± 6.8	6.8±0.42	125±11.6	1.11±0.026	1.48±0.047	56.2±6.65	12
Adrenalectomized Rats									
8. None	All died before 20th day								12
9. Cortisone Acetate 1 mg./day	62	90	47± 5.6	—	107	1.17	1.59	38.2	12
10. DCA—1 mg./day	62	162	164± 6.7	—	408	1.08	1.09	46.8	12
11. 1% NaCl to drink	69	132	90± 6.9	—	439	1.05	1.18	45.2	7
12. Cortisone Acetate +DCA	62	120	96± 6.1	—	100	1.17	1.32	40.5	12
13. DCA+NaCl	66	139	113± 8.6	—	352	1.16	1.12	45.3	10
14. Cortisone Acetate +NaCl	65	110	71± 6.7	—	101	1.11	1.51	63.3	12
Intact Rats									
15. None	58	152	162± 7.0	19.3±1.00	302±15.8	0.94±0.018	1.17±0.030	45± 2.82	13
16. MAD* 2 mg./day	58	147	155± 6.1	19.9±0.45	179±12.0	1.04±0.015	0.89±0.04	307±17.00	12
17. Cortisone Acetate +MAD	57	96	68± 4.1	22.3±1.29	66.7± 6.5	1.15±0.026	1.26±0.063	460±27.82	12
18. Cortisone Acetate +DCA+MAD	59	117	100± 6.6	20.5±1.21	68.0±11.4	1.19±0.026	1.11±0.023	468±27.55	12
19. Testosterone Propionate 0.25 mg./day	60	158	161±10.9	18.7±1.40	167±13.3	0.97±0.010	0.47±0.028	398±40.85	12
20. Cortisone Acetate +Test. Prop.	59	122	106± 7.3	11.7±0.53	87± 6.7	0.98±0.022	0.79±0.041	484±44.82	12
21. Cortisone Acetate+Test. Prop.+DCA	60	133	124± 7.9	10.3±0.24	74± 6.0	1.1 ±0.017	0.65±0.011	511±34.27	12

* MAD = 17α-methyl-5-androstene-3β,17β-diol.

[1] In all cases in this and subsequent tables when combined treatments were used the dose of each was the same as that indicated when the substances were given separately.

The first androgens studied were MAD and TP. They were given initially in daily doses of 2 and 0.25 mgs. respectively in order to achieve approximate equality of androgenicity as judged by the effects on seminal vesicel weight. In such dosage neither MAD nor TP given alone had significant effect on the adrenals of intact animals. Both compounds, however, when given with cortisone helped prevent adrenal atrophy, with MAD being the more effective of the two (Tables 1 and 3). When given in equal doses (Tables 4 and 5) they had equal effects and maintained normal relative adrenal weights and near-normal absolute weights in cortisone-treated rats.

In hypophysectomized rats (Table 2) TP at a 0.25 mg. daily dosage was without effect on adrenal size. MAD (2 mg. per day), on the other hand,

TABLE 2. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN HYPOPHYSECTOMIZED MALE RATS
Experiments of 20 days duration

Group No. & treatment	Body weight—gm.			Organ weights (per 100 gm. body weight)					No. rats
	Initial.	Terminal	% gain	Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
1. Hypx.—none	86	91	+ 5.2	11.7±0.42	228	0.72	0.30	8.2	16
2. Cortisone Acetate 1 mg./day	83	68	−18.1	14.6±0.32	51	0.94	0.31	10.3	19
3. MAD 2 mg./day	85	91	+ 7.0	16.7±0.84	55	0.85	1.02	1025.0	20
4. MAD +Cortisone Acetate	86	71	−17.2	21.6±0.17	41	1.03	1.40	1140.0	20
5. Test. Prop. 0.25 mg./day	88	100	+13.7	12.2±0.74	113	0.73	0.57	1010.0	8
6. Test. Prop. +Cortisone Acetate	85	78	−9.1	18.0±0.87	58	0.95	0.98	1472.0	10

produced a distinct increase in adrenal weight. When cortisone was given in combination with MAD a normal relative adrenal weight was obtained. Actual weights, however, were no greater than when MAD alone was given.

TABLE 3. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT MALE RATS
Experiments of 7 days duration

Group No. & treatment	Body weight—gm.			Food intake/rat/day, gm.	Organ weights (per 100 gm. body weight)					No. rat
	Initial	Terminal	% gain		Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
1. None	64	99	55±1.90	11.0	20.6± .64	350	1.05	.843	14.9	12
2. Cortisone Acetate 1 mg./day	63	70	11±1.40	8.9	11.0± .63	72	1.21	1.044	16.7	12
3. Cortisone Ac.+DCA 1 mg./day	64	81	27±2.83	9.7	10.7± .51	90	1.18	.951	14.4	12
4. Test. Prop. 0.25 mg./day	63	103	62±2.19	11.4	19.5± .68	270	1.09	.618	127.8	12
5. Test. Prop.+Cortisone Ac.	59	78	22±2.30	10.4	15.0± .81	67	1.20	.773	181.1	12
6. Test. Prop+Cortisone Ac.+DCA	56	74	33±2.29	9.3	15.9± .97	61	1.19	.779	182.3	12
7. MAD 2 mg./day	60	93	54±4.43	10.3	22.6±1.53	315	1.057	.723	59.4	12
8. MAD+Cortisone Ac.	60	71	18±2.55	8.9	19.2± .67	56	1.178	.847	84.3	12
9. MAD+Cortisone Ac.+DCA	58	75	31±2.60	9.1	20.0±1.52	67	1.241	.830	92.6	12
10. Estradiol Benzoate 10γ/day	58	82	42±2.92	8.6	23.3± .77	223	1.056	.357	30.1	12
11. Estradiol Benz. +Cortisone Ac.	59	72	20±2.14	8.0	17.4±1.30	84	1.032	.456	36.4	12

It is apparent from Tables 4 and 5 that a sizeable group of other steroids are as effective as MAD or TP in preventing cortisone inhibition of the adrenals. None of these compounds, when given alone, increased adrenal size significantly when compared with control groups observed simultaneously with the possible exception of 17β-hydroxyandrostan-3-one. Some of them may have been expected to depress adrenal weight if given for long periods (Selye and Stone, 1950). Most of the C-19 and C-20 compounds were of approximately equal effectiveness. The 21-carbon ethy, and vinyl derivatives of androstenediol or testosterone were, however, curiously weak or ineffective.

TABLE 4. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT MALE RATS

Experiments of 7 days duration
(Daily dosage: Cortisone 1 mg.; All others 2 mg.;—10 rats per group)

Group No. & treatment	Body weight		% gain	Food intake/rat/day, gm.	Organ weights (100 gm. body weight)				
	Initial	Terminal			Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testes, gm.	Seminal vesicle
1. None	62	97	57±2.0	14.3	20.1±0.56	336	1.13	0.79	14.4
2. *None	58	95	64±3.4	14.3	22.4±0.77	479	1.17	0.70	12.8
3. Cortisone	63	68	12±2.4	11.6	10.3±0.65	63	1.28	1.01	15.8
4. *Cortisone	59	68	15±2.3	12.2	12.3±0.69	47	1.41	0.92	15.8
5. 17β-hydroxyandrostan-3-one	59	94	57±2.8	13.9	23.6±1.01	208	1.13	0.71	164.0
No. 5+Cortisone	61	75	20±2.9	12.0	23.4±1.35	49	1.38	0.89	233.0
6. *17α-methyl-17β-hydroxy-androstan-3-one	62	105	70±3.4	16.0	23.0±1.17	213	1.22	0.56	149.8
No. 6+Cortisone	62	80	31±3.9	14.7	23.3±0.83	50	1.36	0.70	202.0
7. 17α-Methyl-5-androstene-3β, 17β-diol	61	100	60±2.7	14.3	23.6±1.03	224	1.13	0.74	133.0
No. 7+Cortisone	62	77	22±2.6	12.4	22.3±1.02	52	1.30	0.91	174.0
8. 17α-Methyltestosterone	58	93	58±3.8	11.6	23.5±0.90	226	1.09	0.71	166.0
No. 8+Cortisone	59	75	25±2.8	10.0	21.6±0.76	44	1.27	0.90	212.0
9. *17β-methoxy-4-androsten-3-one	60	102	71±1.5	15.2	21.9±6.70	344	1.19	0.65	71.8
No. 9+Cortisone	61	75	24±2.3	13.2	21.5±0.49	60	1.30	0.85	110.3
10. *Testosterone Propionate	58	102	75±3.3	14.6	22.9±0.70	204	1.18	0.67	179.0
No. 10+Cortisone	59	76	30±2.7	13.1	20.8±1.03	49	1.50	0.83	234.0
11. 5-androstene-3β,17β-diol	58	95	59±4.3	13.4	21.2±1.14	299	1.13	0.68	48.2
No. 11+Cortisone	57	70	20±3.0	11.2	20.7±1.51	58	1.26	0.94	68.4
12. 17α-methyl-3β,17β-androstanediol	57	94	62±4.3	13.6	21.7±1.30	214	1.12	0.73	75.2
No. 12+Cortisone	59	72	20±2.3	12.9	20.7±1.22	49	1.26	0.73	105.5
13. Androstane-3α,17β-diol	57	92	60±3.6	13.0	23.3±1.02	213	1.17	0.70	171.8
No. 13+Cortisone	57	71	23±2.0	11.3	19.9±1.33	46	1.32	0.88	213.5
14. 17α-Methylandrostan-17β-ol	62	98	57±3.3	14.1	20.2±0.86	296	1.10	0.54	45.3
No. 14+Cortisone	60	71	18±2.5	11.7	18.0±0.78	49	1.27	0.72	61.3
15. *Dehydroepiandrosterone	62	104	68±2.1	15.9	17.6±0.87	358	1.16	0.73	29.0
No. 15+Cortisone	62	76	23±2.9	13.7	16.1±0.65	74	1.50	0.92	33.6
16. *17α-Ethylandrostane-diol	59	94	60±3.2	14.6	21.9±1.00	368	1.18	0.70	12.7
No. 16+Cortisone	61	71	18±2.4	12.6	15.4±0.48	80	1.31	0.83	17.3
17. 17α-Ethyltestosterone	60	93	62±5.0	13.9	18.8±1.3	287	1.04	0.53	91.6
No. 17+Cortisone	61	73	20±3.0	12.6	14.0±0.85	52	1.22	0.67	127.4
18. 17α-Vinyltestosterone	63	98	58±3.0	13.0	18.9±0.64	309	1.16	0.52	93.1
No. 18+Cortisone	60	74	22±2.9	13.5	12.8±0.77	62	1.22	0.62	129.3
19. 17α-Ethyl-5-androstene-3β, 17β-diol	60	95	56±5.5	13.4	20.8±1.62	323	1.10	0.79	24.3
No. 19+Cortisone	61	72	18±2.3	11.8	12.7±0.97	67	1.21	0.97	32.0
20. *3β-Methoxy-5-androsten-17-one	60	98	62±3.2	14.7	21.9±0.52	360	1.15	0.78	14.8
No. 20+Cortisone	60	72	20±3.4	13.1	12.1±1.03	84	1.32	1.05	20.8
21. 17α-Vinyl-5-androstene-3β, 17β-diol	62	101	56±3.3	14.4	19.7±0.85	327	1.12	0.80	21.8
No. 21+Cortisone	56	68	21±3.5	10.8	11.6±0.88	66	1.17	0.90	23.1

* The groups marked by an asterisk were observed in one continuous experiment and those not so designated in another.

It happened that our first observation of adrenal maintenance in cortisone-treated animals was with 2 mg. daily doses of MAD. For the sake of uniformity most other steroids were compared with MAD at that same dose level. It is apparent, however, from a cursory study with the small number of animals shown in Table 6 that at least MAD and 5-androstene-3β,17β-diol were effective in doses of less than 2 mg. per day. The same result was found by Winter *et al.* (1953). It is also apparent (Table

TABLE 5. SUMMARY OF DATA ON EFFECT OF CORTISONE ALONE AND IN COMBINATION WITH OTHER STEROIDS ON ADRENAL WEIGHT

Treatment[1]	Adrenal weight, mg.	Adrenal weight, mg./100 gm.	No. rats
None	20.3	21.0	32
Cortisone Alone	7.7	11.2	33
Cortisone+the following:			
17β-hydroxyandrostan-3-one (dihydrotestosterone)	17.1	23.4	10
17α-methyl-17β-hydroxyandrostan-3-one (methyldihydrotestosterone)	18.8	23.3	10
17α-methyl-5-androstene-3β,17β-diol (MAD)	17.1	22.3	10
17α-methyltestosterone	16.0	21.6	10
17β-methoxy-4-androsten-3-one (Testosterone methyl ether)	16.1	21.5	10
Testosterone Propionate	15.7	20.8	10
5-androstene-3β,17β-diol	14.2	20.7	10
17α-methyl-3β,17β-androstanediol	15.1	20.7	10
Androstane-3α,17β-diol	14.1	19.9	10
17α-Methylandrostan-17β-ol	12.7	18.0	10
Estradiol benzoate—10γ/day	12.5	17.4	12
Dehydroepiandrosterone	12.0	16.1	10
17α-ethylandrostane-3β,17β-diol	10.8	15.4	10
17α-ethyltestosterone	10.0	14.0	10
17α-vinyltestosterone	9.7	12.8	10
17α-Ethyl-5-androstene-3β,17β-diol	9.3	12.7	10
3β-Methoxy-5-androsten-17-one (Dehydroepiandrosterone methyl ether)	8.5	12.1	10
17α-Vinyl-5-androstene-3β,17β-diol	7.8	11.6	10

[1] All dosages were: 1 mg. Cortisone+2 mg. of other steroids (except Estradiol benzoate) per day for 7 days. The effect of the various steroids given alone, together with other related data, is shown in Tables 3 and 4.

TABLE 6. EFFECT OF 17α-METHYL-5-ANDROSTENE-3β,17β-DIOL (MAD) AND 5-ANDROSTENE-3β,17β-DIOL (AD) ON ADRENAL WEIGHT WHEN GIVEN ORALLY OR IN SMALL SUBCUTANEOUS DOSES

(4 rats per group, treated for 7 days)

Daily treatment	Adrenal weight, mg.	Adrenal weight, mg./100 gm.
2 mg. MAD by stomach tube+1 mg. Cortisone subcu.	9.1	13.8
2 mg. AD by stomach tube+1 mg. Cortisone subcu.	8.3	11.9
1 mg. MAD subcu.+1 mg. Cortisone subcu.	15.2	21.8
1 mg. AD subcu.+1 mg. Cortisone subcu.	13.6	18.5
0.5 mg. MAD subcu.+1 mg. Cortisone subcu.	13.4	20.4
0.5 mg. AD subcu.+1 mg. Cortisone subcu.	10.7	15.7

6) that neither of the two above-mentioned compounds were fully active when given orally in 2 mg. doses. Further studies along this line are in progress.

The effects of small doses of estradiol benzoate were surprisingly like those of the larger doses of androgenic steroids in maintaining adrenal weight in the cortisone-treated animals (Table 3). It is a well-known property of estrogens to cause an adrenal weight increment in intact animals when given alone and that was seen here. Given with cortisone it had an effect on adrenal maintenance intermediate between that of the most active and least active compounds shown in Table 5. Further studies with steroids related to estradiol are now in progress.

Adrenal Histology

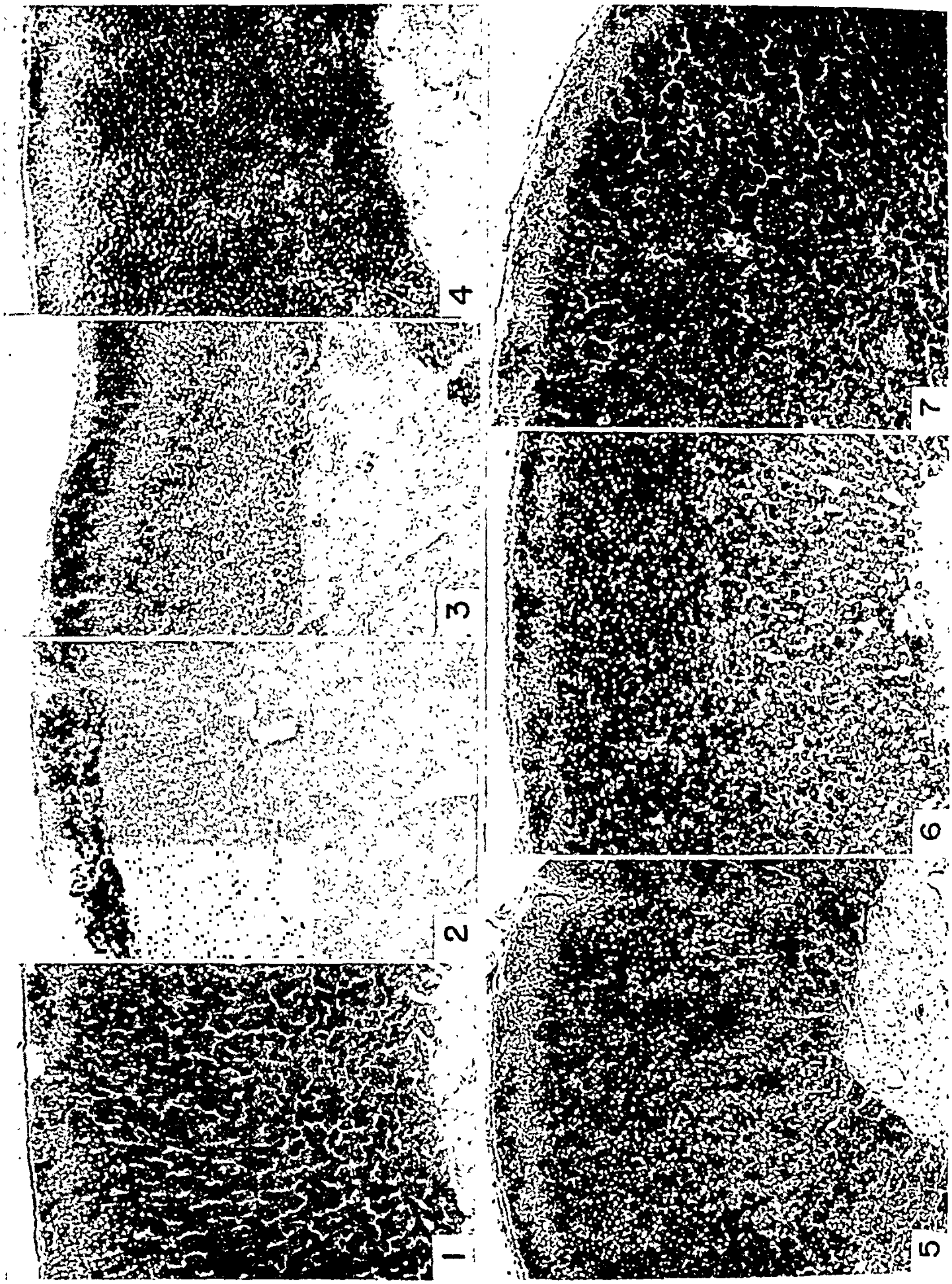
Comprehensive histological study was done only on the adrenals from the animals shown in Table 3 and on those from Table 4 treated with 2 mg. daily of TP and 17β-methoxy-4-androsten-3-one with their appropriate controls. The latter compound was of especial interest because while it maintained adrenal weight as well as TP its androgenic activity was greatly reduced.

a. Lipid: The control glands presented the typical concentration of lipid in the adrenal cortex (Fig. 1). The glomerulosa layers were generally narrow and while most cells contained lipid an occasional cluster lacked it and broke the continuity of a continuous ring under the connective tissue capsule. A sudanophobic zone separated the glomerulosa and fasciculata. This band of lipid free cells apparently is a characteristic of the male rat. The outer fasciculata was fat laden and this heavy concentration in some cases continued to the reticularis or in other cases a gradual decrease in concentration occurred toward and through the reticularis.

Administration of cortisone acetate appeared to favor an enlargement of the glomerulosa emphasized by the intense lipid staining of many of the cells (Fig. 2). An occasional adrenal exhibited some fascicular fat, generally in the outer half, but the staining intensity was always subnormal. In more than half of the glands, the fasciculata and reticularis were lipid free; these tended to be the smaller glands of the series.

The simultaneous administration of 1 mg. per day of DCA with cortisone unexpectedly offered some protection from lipid depletion (Fig. 3). Some fascicular fat was present, ranging from a tinge to a moderate amount but the lipid was always relegated to the outer third or half of the fasciculata. Cortisone seemed to have the dominance in enlarging the glomerulosa but the lipid was not uniformly heavy in that zone.

MAD in 2 mg. daily amounts had no effect on adrenal lipid distribution.



FIGS. 1–7

However, when combined with cortisone the depleting effects of the cortisone were largely prevented and the lipid distribution was within the range of normal limits (Fig. 5). The impression was gained, however, that the glomerulosa was still enlarged by the cortisone but when DCA was added in a triplicate treatment that the glomerulosa returned to normal size in some but not all cases (Fig. 7).

Testosterone proprionate in 0.25 mg. daily amounts whether given alone, with cortisone or with cortisone and DCA had no effects except to inhibit slightly the lipid-depleting effects of cortisone. Two mg. doses of TP and of 17β-methoxy-4-androsten-3-one had effects indistinguishable from those described above with the same dose of MAD (Figs. 4 and 6).

Estradiol benzoate (10γ per day) effectively eliminated the sudanophobic zone (as per Greep and Jones, 1950) but did not alter lipid in other parts of the cortex. When estradiol was given with cortisone the lipid distribution was otherwise normal in four of ten cases. The six remaining cases had enlarged glomerular zones which were fat-laden while other parts of the cortex showed only a uniform hazy film of stain. The pattern was that of a cortisone-treated gland but instead of the fasciculata and reticularis being mostly fat free there was always some lipid.

b. Alkaline phosphatase: Using glycerophosphate as a substrate, the normal adrenal exhibited a considerable concentration of enzyme in the glomerulosa; the nuclei stained markedly and the cytoplasm slightly. Only slight amounts were observed in the nuclei and none in the cytoplasm of the other cortical cells. Cortisone appeared to reduce slightly the fascicular enzyme concentration whereas MAD and TP tended to increase it slightly. The uncertain impression was gained that the increase in alkaline phosphatase imposed by androgen was not altered by simultaneous treatment with cortisone.

c. Cholesterol esters: The Schultz technique, presumably revealing cholesterol or its esters, was postive in the adrenal cortices of all control rats.

←

FIGS. 1–7. Adrenals stained with sudan black B from animals treated with various steroids for seven days. ×176. These figures are described in the text with other related data shown in tables as indicated below. Lipid content essentially normal in Figures 1 and 4 to 7.

1. Control—no treatment (Table 3).
2. Cortisone acetate only (Table 3).
3. Cortisone+DCA (Table 3).
4. Cortisone+17β-methoxy-4-androsten-3-one (Tables 4 and 5).
5. Cortisone+17α-methyl-5-androstene-$3\beta,17\beta$-diol (Table 3).
6. Cortisone+Testosterone Propionate (Tables 4 and 5).
7. Cortisone+17α-methyl-5-androstene-$3\beta,17\beta$-diol+DCA (Table 3).

pounds which did not antagonize cortisone tended to decrease adrenal weight when given alone in our short-term experiments (Table 4). It is of interest that the "blocking" of an active grouping at position 3 of the steroid molecule by making the 3-methyl ether of dehydroepiandrosterone (No. 20 of Table 4) completely eliminated its adrenal-maintaining ability. This chemical maneuver will be more enlightening when tried on other more active compounds because dehydroepiandrosterone (No. 15 of Table 4) antagonized cortisone only weakly and by itself tended to be an adrenal inhibitor. The "blocking" of position 17 of testosterone by making its 17-methyl ether (No. 9 of Table 4), while greatly reducing its androgenic activity (Table 4 and unpublished observations), did not alter its ability to antagonize cortisone.

Correlation with other Biological Properties

No apparent correlation can be made between other biological properties of the steroids tested and their ability to prevent cortisone inhibition of the adrenals. Most of the compounds used were androgens. Some were strong, however, and some very weak as judged by their ability to stimulate the seminal vesicle and not all of the androgens tried would maintain the adrenals. Furthermore, the positive effects of estradiol benzoate in small doses shows that the effect is not limited to the classical androgens. Most of the non-corticoids used inhibited testes but that was true to variable extents and it occurred whether or not the compounds maintained the adrenals. Likewise all of the compounds used tended to antagonize the body growth-inhibiting effects of cortisone irrespective of their effect on the adrenal. It is likely, therefore, that adrenal maintenance is a separable property of a large group of steroids which at the moment have no other obvious common feature.

Growth

It is a common clinical finding that the administration of androgens with cortisone prevents some of the catabolic effects of the cortisone (e.g. Kinsell, 1951; Clark, Manning and Appleton, 1951; Luft and Sjogren, 1951). It is likely that the ability of testosterone and other steroids as seen here to prevent partially cortisone-induced growth inhibition is a manifestation of what is usually called the anabolic action of steroids. If this be true, the animal whose growth is inhibited by cortisone might serve as an assay specimen for such anabolic steroids. In that case, however, it would have to be said the MAD is a weaker anabolic stimulant than TP when given in doses that are of approximately equal androgenicity. Before such conclusions are made, however, our observations would have to be correlated with nitrogen metabolism in pair-fed or force-fed animals and distinction made between weight increment due to salt and water retention and tissue growth. In any case it is of interest that growth in the cortisone-treated animal is maintained better by a combination of DCA and TP than by either substance alone.

The growth-inhibiton and other toxic manifestations of cortisone or ACTH treatment have been in part antagonized by a variety of means such as liver therapy (Ershoff, 1951), aureomycin (Meites, 1952), potassium (Whitney and Bennett, 1952; Glafkides *et al.*, 1952) and fat (Kinsell *et al.*, 1951). Such results plus those of the type reported here with steroids indicate clearly that the continuing search for agents which ameloriate the unwanted effects of cortisone is worthwhile.

SUMMARY

1. A large number of steroids including methylandrostenediol and testosterone propionate prevented the adrenal-inhibitng effect of cortisone in rats as judged by the maintenance of adrenal weight, general histological appearance and the amount and distribution of sudanophilic lipid. Most of the steroids tried which contained 19 or 20 carbon atoms were effective in preventing adrenal atrophy; C-21 steroids were either weak or inactive. Such results with TP and MAD confirm the findings of Winter *et al.* The effects were observed of several steroids in triplicate combinations and of a high salt diet in combination with DCA and cortisone.

2. The majority of the compounds which prevented cortisone inhibition of the adrenals possessed some androgenic activity but their effectiveness was not correlated with their relative androgenicity and all androgenic compounds did not prevent adrenal atrophy. Estradiol in small doses also had to some degree the ability to antagonize the adrenal-depressing effects of cortisone.

3. Compounds which were effective in preventing cortisone from causing adrenal atrophy had little, if any, adrenal-stimulating effect when given alone.

4. Cortisone was given in a dose of 1 mg. per day and most other steroids, either alone or in combination with cortisone, at a dose of 2 mg. per day. Preliminary experiments, however, suggested that methylandrostendiol and presumably other steroids can prevent cortisone-induced adrenal atrophy at a much smaller daily dose than 2 mg. when injected but that its effectiveness is reduced by oral administration. Estradiol benzoate had some activity in 10γ doses.

5. In hypophysectomized rats methylandrostenediol given alone partially prevented adrenal atrophy. When given with cortisone, relative but not absolute adrenal weights were enhanced. Cortisone alone increased the relative but not absolute adrenal weight after hypophysectomy. The

conclusion is not yet warranted that the adrenal-maintaining ability of combinations of steroids is independent of the pituitary since a given treatment was found to be more effective in intact than in hypophysectomized animals.

6. Cortisone depleted the sudanophilic lipids of the fasciculata and reticularis, an action which was blocked by methylandrostenediol, testosterone propionate and 17β-methoxy-4-androsten-3-one. The latter compounds were without effect on sudanophilia when given alone. Estradiol benzoate eliminated the sudanophobic zone of the male rat adrenal and partially inhibited the lipid-depleting effects of cortisone. More lipid was present in glands of animals treated with cortisone plus DCA than in those given cortisone only. A different pattern of results was obtained, however, when adrenals were stained by the Schultz technique for cholesterol or its esters.

7. Testosterone propionate, DCA and most of the other steroids used, including estradiol benzoate, antagonized to some degree the body weight-inhibiting effects of cortisone whether or not they prevented adrenal atrophy. In some but not all groups, the animals on combined therapies ate more food than those on cortisone alone.

8. The normal weight increment of the testes and the kidneys was inhibited less by cortisone than that of the body as a whole.

9. It is suggested that the ability to prevent cortisone inhibition of the adrenal defines a group of steroids without other common biological properties.

ADDENDUM

Work completed since the foregoing was written has established the following: (1) Judged by its effects on adrenal weight and maintenance of sudanophilic lipids at low dosage (0.25 mg. per day) MAD is the most effective steroid in preventing adrenal atrophy of the several compounds which seemed equally active at high dosage (2 mg. per day) in Table 5. (2) The following were either weak or inactive at 2 mg. daily doses: progesterone, ethinyltestosterone, pregnenolone, 3β-methoxy-5-pregnen-20-one, and 4-androsten-3-one. The 3-methyl ethers of MAD and androstenediol were slightly less active than the latter compounds themselves. Such results, together with those recorded in the above paper, show that some functional group at C-17, best exemplified by a β-hydroxyl and probably enhanced by the presence of an α-methyl group, is necessary for adrenal-maintaining activity. The weakness or inactivity of C-21 compounds is confirmed. Alterations in functional groups at C-3, or their absence, result in quantitative variations of, but not loss of, activity. (3) Further work with estradiol benzoate, α- and β-estradiol and stilbestrol indicates that they exert only a weak and inconstant activity which unlike the more effective steroids is probably due to their stress-like stimulation of the adrenal.

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ANEMIA IN THE RABBIT DURING PREGNANCY AND FOLLOWING TREATMENT WITH WATER SOLUBLE OVARIAN EXTRACTS[1,2]

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ANEMIA of pregnancy has been described in a number of widely divergent species of mammals. In general it has been concluded that this phenomenon is due to a hemodilution (Bond, 1948; Houssay, 1951), although the etiologic factors responsible for the anemia remain unknown. Some investigators still feel that the anemia may be due to nutritional deficiencies. Forman (1950) has reported excellent results in pregnant women with a molybdenum iron complex, which caused an increase in the hemoglobin and prevented the anemia of pregnancy. However the work of Mitchell and Miller (1931), Beard and Myers (1931) and Van Donk, Feldman and Steenback (1934) on the rat, would seem to indicate that this anemia is not due to a lack of nutritional factors.

The ability of different hormones or the lack of these hormones to cause an anemia has been reported rather extensively. It has been shown that hypophysectomy, throidectomy or treatment with estrogens depress the number of circulating erythrocytes (see review by Gordon and Charipper, 1947). Recently Witten and Bradbury (1951) reported a hemodilution and decrease in the red blood cell count in women after treatment with estrogen. Studies in the rat indicate that the placenta is the source of the factor (or factors) responsible for the anemia of pregnancy (Newcomer, 1947), and that the anemia is due to a hemodilution (Bond, 1948). These results would tend to suggest that the estrogens, which are present in increasing amounts during pregnancy, may be the etiologic agents.

The present investigation was undertaken to elucidate the factors responsible for the anemia of pregnancy. In the initial phase of this study, the normal pattern of pregnancy was followed by determining the changes in the water balance, the circulating erythrocytes, leucotyces, hemoglobin, hematocrit, non-protein nitrogen and chlorides of the blood and the urinary chorides. In the second part of this study the same determinations

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[1] This investigation was started at the Biological Laboratories, Harvard University, Cambridge, Mass.

[2] Aided in part by grants from the Thompson Science Fund, Boston, Massachusetts and the Chilcott Laboratories, The Maltine Co., Morris Plains, New Jersey.

were made in rabbits during treatment with a water soluble extract of the ovary of pregnant sows. This preparation (relaxin) was tested in these experiments since previous work (Zarrow 1949) had shown that it possessed the ability to cause water retention and thus possibly produce a hemodilution.

MATERIAL AND METHODS

Grey, chinchilla rabbits obtained from Rockland Farms were used in the present study. All animals were housed under normal conditions of constant temperature and humidity with free access to Purina rabbit chow, lettuce and water. During the course of the experiments some of the rabbits were placed in metabolism cages and daily measurements made on their water intake and urine output. Blood samples were obtained from the marginal vein of the ear at regular intervals of 5, 10, 15, 20, 25, and 30 days after mating and at 5 and 15 days after parturition. In the experiments involving treatment with ovarian extracts, blood samples were obtained every third day and a minimum of three control samples were obtained prior to mating or treatment.

Total erythrocyte and leucocyte counts were carried out in duplicate and hemoglobin concentrations were determined in a Klett photoelectric colorimeter after dilution with 0.1 N hydrochloric acid. Reticulocyte counts were made using brilliant cresyl blue and a minimum of 1,000 red blood cells were counted. Hematocrit determinations were carried out in Wintrobe tubes after centrifugation for 30 minutes at 2,500 R.P.M. Non-protein nitrogen determinations of the blood were made according to the micro-Kjeldahl method using sulfuric and phosphoric acid for digestion and determining the ammonia colorimetrically after direct Nesslerization (Hawk, Oser and Summerson, 1947). The serum chlorides were determined according to the method of Shales and Shales (1941) by titrating with a standard mercuric nitrate solution and using diphenyl carbazone as an indicator. Urinary chlorides were determined as above except that the urine was first treated with norit and 10 per cent sodium nitro-prusside was used as the indicator.

Several different relaxin preparations[3] were used in this study as the source of the water soluble factor. In all instances the extract was obtained from the ovaries of pregnant sows and purified for its relaxin content. Consequently the amount of ovarian ovarian extract used in these experiments is expressed in guinea pig units (GPU) of relaxin.

OBSERVATIONS AND RESULTS

Blood Constituents During Pregnancy

Data on the hemoglobin and cellular elements of the blood of the normal, pregnant and post partum rabbit may be seen in Table 1. The normal rabbits of this strain possess a red blood cell count of 5,950,000 per cmm. A highly, significant decrease in the circulating erythrocytes is seen by the 20th to the 25th day of pregnancy. The decline is progressive throughout

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[3] Relaxin preparation P XXVII-5 was obtained through the courtesy of Dr. E. H. Frieden and several other relaxin preparations (Releasin) were obtained from Dr. Kroc, Chilcott Laboratories. Preparation J46 was prepared by one of us (MXZ) and has been used in other studies involving the action of relaxin (Zarrow and Money, 1948; Zarrow, 1949).

TABLE 1. CHANGES IN THE CELLULAR COMPONENTS AND HEMOGLOBIN OF THE BLOOD OF THE RABBIT DURING PREGNANCY[1]

	Erythrocytes, million per cmm.	Hb., gm.%	Hematocrit, %	Reticulocytes, %	Color index
Days pregnant					
0	5.95 ± 0.083[2]	11.9 ± 0.22	38.7 ± 0.69	2.1 ± 0.24	1.00[3]
5	5.85 ± 0.085	11.9 ± 0.32	39.0 ± 0.87	2.2 ± 0.36	1.02
10	5.70 ± 0.10	11.8 ± 0.28	38.0 ± 0.84	2.6 ± 0.35	1.04
15	5.43 ± 0.14	11.7 ± 0.21	37.0 ± 1.20	3.9 ± 0.42	1.07
20	5.12 ± 0.14	11.3 ± 0.22	35.3 ± 1.36	6.8 ± 1.03	1.09
25	5.00 ± 0.18	10.8 ± 0.31	33.4 ± 1.45	3.8 ± 0.72	1.07
30	4.71 ± 0.12	9.9 ± 0.34	32.1 ± 0.34	2.4 ± 0.43	1.03
Days post partum					
5	5.09 ± 0.18	10.2 ± 0.40	33.2 ± 0.40	5.3 ± 0.36	1.00
15	5.81 ± 0.088	11.4 ± 0.32	34.3 ± 1.01	2.2 ± 0.16	1.01

[1] Ten rabbits were used and a total of 12 pregnancies studied.

[2] Standard error $= \sqrt{\dfrac{\Sigma d^2}{N(N-1)}}$

[3] $\dfrac{\text{gm. Hb/100 ml. pregnant rabbit blood}}{\text{gm. Hb/100 ml. normal rabbit blood}} \div \dfrac{\text{RBC count of pregnant rabbit}}{\text{RBC count of normal rabbit}}$

gestation with a return to normal levels after parturition. As early as 5 days postpartum the number of circulating red blood cells has returned to the normal value. The average decline in erythrocytes is approximately of the order of 20 per cent. Along with the drop in red blood cells may be seen a concommitant decline in hemoglobin and hematocrit. All three appear to parallel each other throughout gestation with the most rapid drop occurring between the 20th day and the termination of pregnancy. Complete recovery in the hemoglobin concentration may be noted by the 15th day after delivery. The color index also indicates the parallel nature of the changes in the red blood cell count and hemoglobin concentration. The hematocrit showed a drop of 17% by the 30th day of pregnancy and was still 11% below normal 15 days after parturition.

A rise in reticulocytes may be seen by the 15th day of pregnancy with a peak on the 20th day. In spite of the increasing severity of the anemia the reticulocytes returned to normal just prior to partutition and showed a second rise on the 5th day postpartum.

Determination of the non-protein nitrogen of the blood indicated a marked and progressive drop throughout the course of pregnancy. The first significant change was noted on the 20th day of pregnancy when the concentration decreased from a normal of 42.6 mg. % to 33.4 mg.%. The decline continued throughout pregnancy with partial recovery by the 5th day postpartum and a return to normal by the 15th day. Serum chlorides failed to indicate any significant changes during pregnancy or the postpartum period. The urinary chlorides showed a steady decline in the rate of excretion throughout the course of pregnancy and a return to normal by the 5th day post partum (Table 2). However, it is difficult at this time to state whether the drop was significant due to the high standard errors obtained with the urinary chlorides. Figure 1 shows the entire sequence of events during pregnancy in a normal rabbit and indicates the correlation in time for changes in the various blood components.

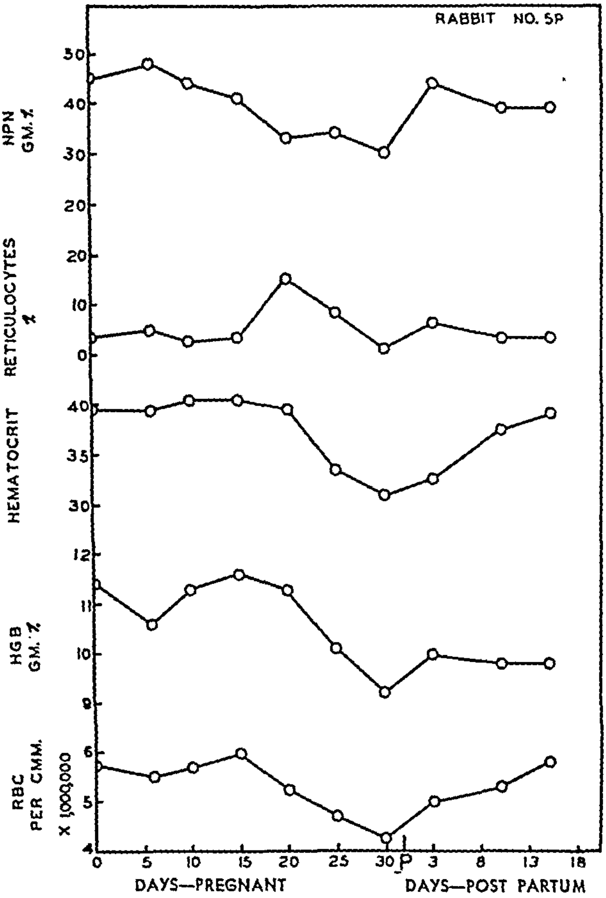

FIG. 1. Changes in the number of circulating erythrocytes, reticulocytes, hematocrit, hemoglobin concentration and non-protein nitrogen of the blood of Rabbit No. 5P during pregnancy and following parturition.

Analysis of the type of anemia present from data on several rabbits indicate that it was normochromic with a slight macrocytosis present. The mean corpuscular volume and mean corpuscular hemoglobin concentration showed no marked changes. A slight microcytosis occurred during recovery in the postpartum period.

TABLE 2. CHANGES IN THE SERUM NON-PROTEIN NITROGEN AND THE SERUM AND URINARY CHLORIDES DURING PREGNANCY IN THE RABBIT[1]

	Serum, non-protein nitrogen, m.%	Chlorides	
		Serum, milli-equivalents	Urine, gm. per 24 hours
Days pregnant			
0	42.6 ± 1.7[2]	105 ± 1.1	0.867 ± 0.177
5	44.5 ± 1.7	104 ± 1.3	—
10	42.4 ± 1.5	104 ± 1.1	0.750 ± 0.245
15	40.2 ± 1.9	102 ± 1.8	—
20	33.4 ± 2.1	104 ± 1.2	0.620 ± 0.34
25	32.6 ± 1.6	104 ± 1.1	0.566 ± 0.332
30	30.4 ± 1.5	107 ± 0.91	0.472 ± 0.307
Days post partum			
5	37.0 ± 1.4	105 ± 1.2	0.831 ± 0.284
15	40.0 ± 1.1	105 ± 0.98	0.906 ± 0.198

[1] Eight rabbits were used and a total of 10 pregnancies studied.

[2] Standard error = $\sqrt{\dfrac{\Sigma d^2}{N(N-1)}}$

Water Balance During Pregnancy

The water intake and urine output was measured in a total of 18 pregnancies involving 15 rabbits. Throughout this study the animals were kept in metabolism cages and received food and water *ad libitum.* No lettuce

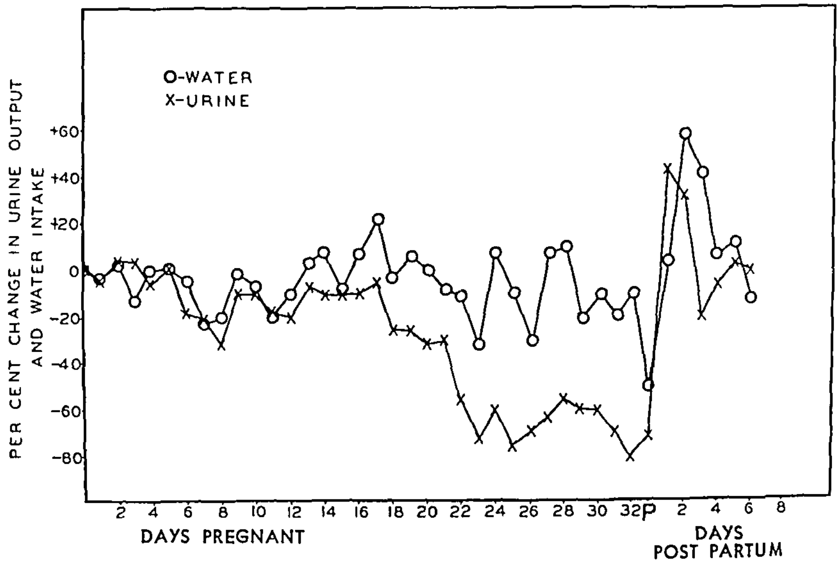

FIG. 2. Comparison of the urine output and water intake during pregnancy in rabbit No. 28. Both volume of urine and water are plotted as a per cent increase or decrease from the pre-mated levels. The decrease in urine output preceded the slight change in water intake and was much more marked.

was permitted the animals during the course of these experiments. All of the animals went into a positive water balance during the 2nd half of pregnancy but the degree of antidiuresis varied with the individual.

Figure 2 shows the data obtained on water balance studies during pregnancy in rabbit No. 28. Both the water intake and the urine output are plotted as a percentage change from the average values obtained prior to mating. Thus it is possible to plot the data on the same ordinate and to make a direct comparison of water balance. It is obvious that while the urine dropped markedly, the per cent decrease in water intake was not as great. Actually rabbit No. 28 showed only a small drop in water intake. In other rabbits a more marked decrease in water intake was obtained but in all instances the urine retention appeared first and was much more marked, indicating a definite retention of water.

Blood Constituents Following Treatment with Ovarian Extract

Data on the hemoglobin, hematocrit and cellular elements in the blood of the rabbit during treatment with relaxin may be seen in Table 3. A total of 8 rabbits were injected with 250 G.P.U. of the ovarian extract (assayed for its relaxin activity) three times daily for 12 days. The rabbits showed a normal erythrocyte count of 5,840,000 cells per cu. mm. Within six days after the start of treatment the count dropped to 5,310,000 and continued to drop so that by the 12th day the count had fallen to 4,546,000,

TABLE 3. CHANGES IN THE CELLULAR COMPONENTS AND HEMOGLOBIN OF THE RABBIT AFTER TREATMENT WITH 250 G.P.U. OF RELAXIN, 3 TIMES DAILY FOR 12 DAYS

	Erythrocytes, million per cu. mm.	Hemoglobin, gm. %	Hematocrit, %	Reticulocytes, %	Color index[1]
Length of treatment days					
0	5.84 ±0.25[2]	12.4 ±0.28	40 ±0.77	2.8 ±0.43	1.00
3	5.50 ±0.14	11.1 ±0.25	38 ±1.5	3.3 ±0.53	0.94
6	5.31 ±0.21	10.5 ±0.43	35 ±1.8	4.5 ±0.96	0.93
9	5.00 ±0.27	9.4 ±0.34	32 ±1.0	5.6 ±1.06	0.89
12	4.56 ±0.28	9.1 ±0.25	30 ±1.1	5.9 ±1.03	0.94
Days after cessation of treatment					
3	5.04 ±0.42	9.8 ±0.37	32 ±1.4	7.3 ±0.62	0.92
6	5.18 ±0.34	10.3 ±0.36	35 ±1.6	7.6 ±0.52	0.94
9	—	10.8 ±0.38	36 ±1.4	5.5 ±1.77	—
24–28	5.80 ±0.29	11.5 ±0.24	39 ±0.61	2.7 ±0.70	0.93

[1] $\frac{\text{gm. Hb./100 ml. (treated rabbit blood)}}{\text{gm./Hb./100 ml. (normal rabbit blood)}} \div \frac{\text{RBC count (treated rabbit)}}{\text{RBC count (normal rabbit)}}$

[2] Standard error $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

a decrease of 20.9%. A rise in the circulating red blood cells occurred within three days after the cessation of treatment with a return to the normal count by four weeks. A 25% drop in the hematocrit and 26.6% drop in the hemoglobin occurred concomitantly with the decrease in the circulating erythrocytes. The decline was progressive throughout the course of the treatment; the hemoglobin dropped from a normal of 12.4 gm % to 9.1 gm % and the hematocrit from 40 to 30. In both instances a rise was observed by the 3rd day after the cessation of treatment and the levels were back to normal 24 days after treatment had stopped.

The color index showed a slight but insignificant drop during treatment with the ovarian extract. In general, the color index indicated the parallel nature of the drop in hemoglobin concentration and circulating erythrocytes. The reticulocytes increased progressively throughout the course of treatment from a normal of 2.8% to 5.9%. During the first 6 days after cessation of treatment the reticulocytes continued to increase reaching an average concentration of 7.6%. Thereafter the number of cells declined to a normal level by the 24th day.

Table 4 contains the data on the changes observed in the non-protein nitrogen of the blood and in the serum and urinary chlorides. The most significant changes were observed in the concentration of the non-protein nitrogen which dropped from a pretreatment level of 40 mg. per cent to 33 mg. per cent by the 6th day of treatment with the ovarian extract and to 29 mg.% by the 12th day. Within three days after the cessation of treatment the nonprotein nitrogen rose to 36 mg. per cent and by 9 days had returned to normal. No particularly significant changes were noted in the serum chlorides. Normal rabbits in this experiment had an average

TABLE 4. CHANGES IN THE NON-PROTEIN NITROGEN OF THE BLOOD AND IN THE BLOOD AND URINARY CHLORIDES AFTER TREATMENT WITH 240 G.P.U. OF RELAXIN, 3 TIMES DAILY FOR 12 DAYS

	Non-protein nitrogen, mg. %	Chlorides	
		Serum, milli-equivalents	Urine, gm. per 24 hours
Treatment, days			
0	40 ±2.8	103 ±2.2	0.689 ±0.079
3	34 ±2.9	101 ±3.0	0.859 ±0.210
6	33 ±3.0	104 ±2.1	0.390 ±0.014
9	31 ±2.1	106 ±0.9	0.417 ±0.068
12	29 ±1.8	107 ±1.2	0.519 ±0.128
Days after treatment			
3	36 ±1.7	104 ±2.2	1.172 ±0.338
6	38 ±2.2	105 ±1.4	0.840 ±0.221
9	41 ±1.6	104 ±2.2	—
28	42 ±1.7	101 ±2.3	0.669 ±0.073

serum chloride of 103 ±2.2 m. eq. which rose to 107 ±1.2 m. eq. This slight increase is not significant though further studies are needed before an effect on serum chlorides can be ruled out. Urinary chlorides were determined on a limited number of rabbits and the results were highly variable. The normal rabbits showed a urinary chloride excretion of 0.68 ±0.079 gm. per 24 hours. This fell to 0.390 ±0.014 gm. per 24-hours after 6 days of treatment with relaxin and then rose to 0.519 ±0.128 gm.

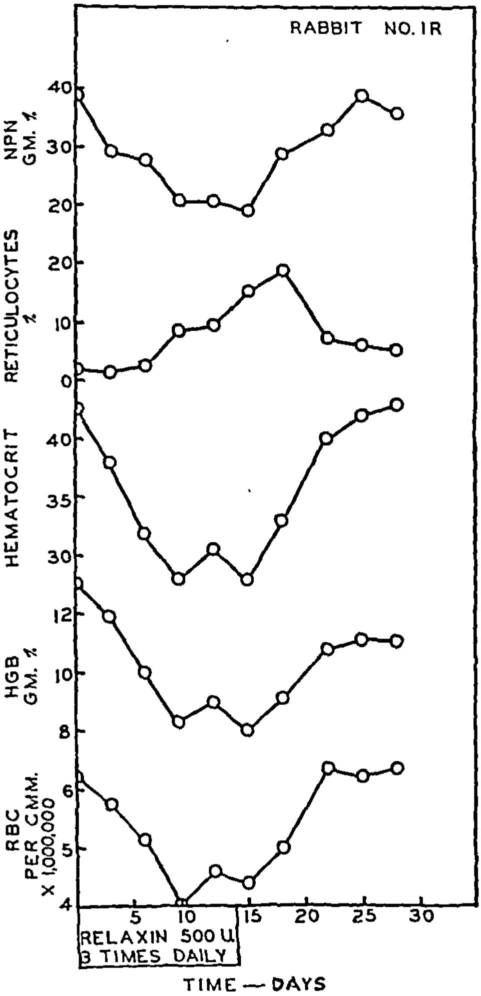

FIG. 3. The effect of a water soluble ovarian extract (relaxin) on the non-protein nitrogen, reticulocytes, hematocrit, hemoglobin, and erythrocytes of the blood of a normal rabbit.

per 24-hours on the 12th day of treatment. On the third day after treatment the excretion rose to 1.172 ± 0.338 gms. per 24-hours and then returned to normal by the 24th day.

Rabbit No. 1R was injected with 500 relaxin G.P.U. of ovarian extract three times daily for 14 days (Fig. 3) and showed the same pattern as noted for the lower dose except for a more acute response. This would tend to indicate that some type of a dose-response relationship exists. A sharp, and parallel decline in the circulating erythrocytes, hemoglobin and hematocrit may be observed. The maximum response occurred on the 10th day, although treatment was continued for 4 more days. Immediately upon the cessation of treatment all values began to return to normal. The nonprotein nitrogen showed a marked drop of 48%, i.e. from 39 gm. % to 20. The reticulocytes showed a marked increase on the 10th day of treatment and continued to rise in the post treatmént period reaching a peak of 18% on the 4th day after treatment was stopped.

It may be noted that no marked changes occurred in either the mean, corpuscular hemoglobin or mean, corpusculr hemoglobin concentration. A slight drop in the mean corpuscular volume occurred but this was only of the order of 5% and its significance is highly questionable. It would appear that the anemia produced by the ovarian extract is both normochromic and normocytic.

Water Balance During Treatment With the Ovarian Extract

Figure 4 shows the results obtained on the water intake and urine output following treatment with 500 G.P.U. relaxin (ovarian extract) for 12 days. The data are expressed as a per cent change from the pretreatment levels and are plotted on the same ordinates. It is obvious from the results that, while both the water intake and urine output drop, the decrease is much more marked with regard to the urine. Furthermore, the decrease in urine output occurred several days prior to the decrease in water intake. Hence the initial effect of the extract on the water balance would appear to be a water retention.

DISCUSSION

It is apparent from these results that the rabbit in common with several other species develops an anemia during pregnancy. The erythrocytes decreased from an average of 5.95 ± 0.083 million per cu. mm. to 4.71 ± 0.12 million per cu. mm. This is a 20% drop in the circulating erythrocytes which is paralleled by a concomitant drop in hemoglobin (16.7%) and hematocrit (17%). The anemia is normochromic with a slight tendency toward macrocytosis. The parallel nature of the decrease in erythrocytes

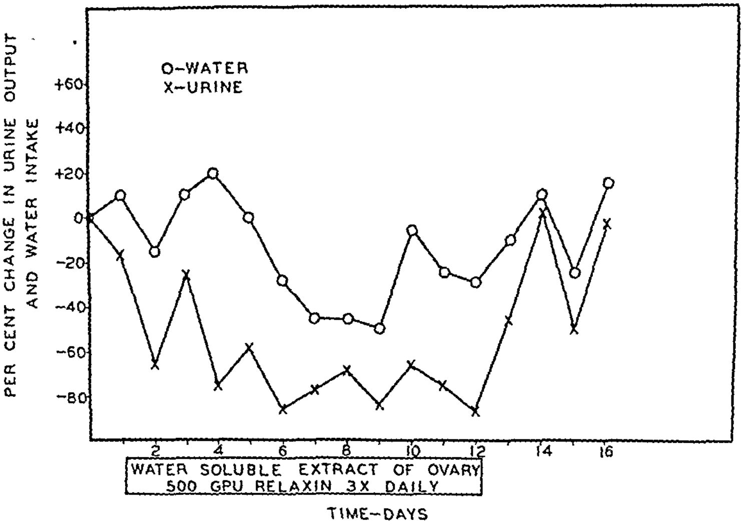

FIG. 4. Changes in water intake and urine output in Rabbit No. 94 after treatment with a water soluble extract of the ovary of the pregnant sow. The data are plotted as a per cent change from the pretreatment levels and indicate a decrease in both water intake and urine output. However the decrease in urine is more marked and occurs first indicating a water retention.

and hemoglobin is also indicated by a lack of change in color index during pregnancy. The reticulocytes increased from a normal of 2.1% to 6.8% on the 20th day of pregnancy and then fell in spite of the increasing severity of the anemia. A second peak in reticulocytes was seen during the recovering phase i.e. on the 5th day after delivery. No significant changes were noted in the white blood cell count or differential.

Treatment with the ovarian extract (250 G.P.U.) for 6 and 12 days induced changes of the same order of magnitude as seen during pregnancy. The 6-day treatment caused a 25.8% drop in the nonprotein nitrogen, 16.9% drop in the erythrocytes and a 21.9% drop in the hematocrit. Treatment for 12 days with the same dose caused a drop of 29.6% in the erythrocytes, 23.1 in the hemoglobin and 24.4 in the hematocrit. Doubling the dose of the ovarian extract increased the severity of the anemia, resulting in a 36.7% drop in the erythrocytes, 36.1% in hemoglobin and 33.9% in hematocrit.

It is of interest to note that the ovarian extract was able to produce a drop in the nonprotein nitrogen of the blood. The decrease was approximately of the same order of magnitude as seen during pregnancy i.e. a 29.5% drop during pregnancy and a 25.8 to 36.7% drop following treatment with the ovarian extract. While disturbances in protein metabolism during pregnancy have been observed by others (Lindeboom, 1948;

Hamilton and Higgins, 1949), it is intriguing to note that the ovarian extract was not only able to produce an anemia but also produce a drop in the nonprotein nitrogen of the blood.

The present results indicate that during pregnancy there is a disturbed water balance. In spite of the decreased water intake in some rabbits, all of them showed some degree of water retention during the final trimester of gestation. Such a condition has been described for women (Chesley, 1944) with an indication of changes in the ratio of extravascular fluid volume and plasma volume (Caton, Roby, Reid and Gibson, 1949). Recently van Dyke (1950) has questioned the antidiuretic action of the ovarian extract used in this study (Zarrow, 1949) on the grounds that the antidiuretic response was the result of a reduced water intake. While a reduced water intake is apparent during pregnancy and also during treatment with the ovarian extract, nevertheless the reduction in urine is far greater, resulting in a marked water retention. Furthermore the decrease in urine output consistently preceded the drop in water intake. This is apparent in Figures 2 and 4 which show the per cent change in the urine and water. The ability of the ovarian extract to produce an anemia, a drop in nonprotein nitrogen, and a positive water balance plus the similarity in the pregnancy-anemia and the extract-induced-anemia would tend to identify this ovarian extract as the possible etiologic factor.

Though relaxin preparations were used in this investigation as the ovarian extract it is apparent that the anemia-inducing effect is not due to relaxin. A number of highly purified preparations of relaxin failed to show any water retention or anemia-inducing action. Recently Frieden and Hisaw (1950) suggested that relaxin may exist as a complex molecule which retains different physiological activities after splitting. Further work is needed along these lines especially in the purification of the different water soluble factors.

SUMMARY AND CONCLUSIONS

Studies were made on the erythrocyte count, leucocyte count, differential count, reticulocytes, hemoglobin concentration, non-protein nitrogen, blood and urine chlorides and water balance of the grey chinchilla rabbit throughout pregnancy and after treatment with water soluble extracts of the ovary. During pregnancy the rabbits developed a normochromic, slightly macrocytic type of anemia, with a 20% drop in erythrocytes a 16.7% drop in hemoglobin and a 17% drop in hematocrit. The reticulocytes showed a sharp peak on the 20th day of pregnancy in spite of the increasing severity of the anemia. With the onset of the anemia, the rabbits developed a definite water retention. The non-protein nitrogen of the blood fell during pregnancy and recovered shortly after parturition. The chloride secretion in the urine dropped though no marked changes were noted in the blood chlorides. It may be concluded that the rabbit develops an anemia of pregnancy concomitant with a water retention.

Treatment with the ovarian extract produced a definite retention of water and an anemia similar to that noted during pregnancy. The minimal dose used in these studies produced a drop of 21% in the circulating erythrocytes, a 23% drop in the hemoglobin, a 24% drop in the hemocrit, and a 30% drop in the nonprotein nitrogen of the blood. The animals showed a marked reticulocytosis and a tendency to chloride retention. Treatment with twice the above amount of ovarian extract, produced a more severe anemia and a greater drop in the nonprotein nitrogen of the blood. Both doses induced a positive water balance with a definite retention of water in spite of a slight decrease in water intake. No marked changes appeared in the serum chlorides. It is suggested that the anemia may be caused by a hemodilution due to an action of the water soluble extract of the ovary and that the anemia-inducing action of the ovarian extract is not due to relaxin.

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PITUICYTES AND THE ORIGIN OF THE ANTIDIURETIC HORMONE[1]

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INTRODUCTION

THE question of the secretory activity of the tissue elements in the pars nervosa and more specifically of the pituicytes has been treated by most authors (Pickford, 1945; O'Connor, 1947; Harris, 1948; Collin and Stutinsky, 1949) as an open one, since the cells of the neurohypophysis do not possess cytological characteristics peculiar to gland cells, and since past attempts to link the production of posterior lobe hormones to microscopically visible changes in the pituicytes had failed. Gersh (1938, 1939) and Gersh and Brooks (1941), for instance, believed that they had shown a relationship between the state of dehydration of their experimental animals and the presence in the pituicytes of granules which they could stain with osmic acid. However, Hickey, Hare and Hare (1941), De Robertis and Primavesi (1942), and Desclin (1947) were unable to confirm these findings. The observations of Wang (1938) which have been frequently cited as supporting the concept of a secretory function of the pituicytes were based on sections stained with the Hortega method which is not suitable for the study of the cytology of secretion.

A new approach to this problem was suggested by the work of Bargmann (1949 a,b), Bargmann and Hild (1949), Bargmann, Hild, Ortmann, and Schiebler (1950), Ortmann (1950, 1951), Hild (1951 a,b; 1952), Kratzsch (1951), Hild and Zetler (1951 a,b; 1952 a,b; 1953 a,b) who demonstrated by histological and experimental methods the presence in the neurohypophysis of material which can be selectively stained with chrome-alum hematoxylin and which is invariably associated with the vasopressor, antidiuretic, and oxytocic hormones. If, as Ranson and his coworkers believed (Fisher, Ingram and Ranson, 1938; Ranson, Fisher and Ingram, 1938; Magoun, Fisher and Ranson, 1939; Ranson and Magoun, 1939), the pituicytes are concerned with the elaboration of the posterior lobe hormones, these elements should be implicated in the production of the stainable material. This question has been answered by Ortmann (1950,

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[1] This work was done under contract No. N6onr-231 between the Office of Naval Research and the University of Colorado.

1951), Hild (1951 a,b; 1952) and Kratzsch (1951) who found no evidence to indicate that the pituicytes produce the stainable substance with which the posterior lobe hormones are associated.

However, Pickford (1952) as well as the investigators who participated in a meeting of the Society for Endocrinology devoted to the "Posterior Pituitary Gland" (Lancet, July 5, 1952, pp. 22–23) do not refer to the evidence offered by Ortmann, Hild and Kratzsch, but continue to base their conclusions on the hypothesis of Ranson.

Although we feel that the data presented by Ortmann, Hild and Kratzsch are valid, we restudied the problem in rats which had been subjected to dehydration both by withholding of drinking water, and by administration of a salt load. Under such conditions the demand for the antidiuretic hormone is greatly increased, since the animals rapidly use it up in an effort to conserve water (Gilman and Goodman, 1937). The pituicytes, if they have a secretory function, should consequently be at the height of their activity in animals whose store of antidiuretic hormone has been depleted by dehydration. In addition, the pituicytes should be more clearly visible in the neurohypophyses of dehydrated animals since the pars nervosa of animals so treated shows a marked depletion of the stainable material. Any sign of secretory activity on the part of the cells should consequently be easily discernible.

MATERIAL AND METHODS

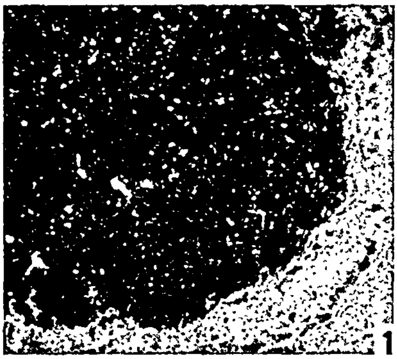
White rats of the Sprague-Dawley and University of Denver Strains were dehydrated in one of two ways: Some received no drinking water for periods up to 13 days, others were given a 2.5% solution of sodium chloride instead of drinking water for 13 days. The period of dehydration by administration of salt had been previously determined in several animals as one resulting in maximum depletion of stainable material in the posterior pituitary. Following the period of dehydration, the animals were given free access to tap water and then sacrificed at regular intervals.

It is important, as Rothballer (1952) has shown, that the rats be killed without allowing them to become unduly excited. Unspecific stress such as that brought about by prolonged handling or by the prick of a hypodermic needle in the administration of an anesthetic leads to the release of antidiuretic hormone within a few minutes. Therefore, the rats were taken from their cages and immediately transferred to a jar containing chloroform.

Fixation of the tissues was accomplished by perfusion through the heart with Zenker-Formol. The pituitaries were embedded in paraffin, sectioned at 5 micra, and stained with Gomori's chrome-alum hematoxylin phloxin (Gomori, 1941; Bargmann, 1949 a,b).

EXPERIMENTS AND OBSERVATIONS

The need for water conservation by the "intact" organism, which occurs in water deprivation, is a stimulus for the release of the antidiuretic hormone (Gilman and Goodman, 1937). In dehydrated animals, the amount of antidiuretic hormone in the posterior lobe is consequently reduced (Hild and Zetler, 1952 b). Histologically, this reduction of hormone content expresses itself in the disappearance of a substance which stains selectively blue with Gomori's chrome hematoxylin-phloxin (Figs. 1 and 2) and to which the antidiuretic hormone is closely bound (Ortmann, 1951; Hild and Zetler, 1952 b). The extent to which hormone content and stainable material are reduced is directly related to the severity of the dehydration. Animals with maximal reduction of hormone content of the posterior lobe show a nearly complete depletion of the stainable substance. When, after reaching this stage, animals are again given drinking water, the stainable material reappears in the posterior pituitary (Figs. 3 to 5). If the pituicytes are concerned with the elaboration of this material, it should first become visible in these cells.



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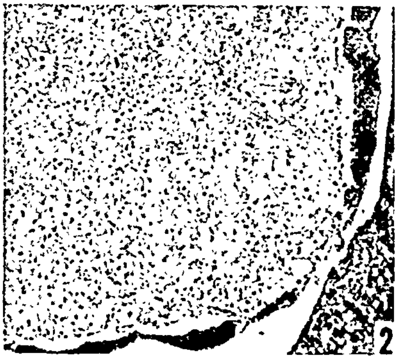

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FIG. 1. Pituitary of a normal rat. The pars nervosa is filled with material staining deep blue. Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxylin phloxin method, photomicrograph. ×60.

FIG. 2. Pituitary of a rat dehydrated by the ingestion of 2.5% sodium chloride solution for 13 days. The pars nervosa is depleted of stainable material. Technique and magnification as in Figure 1.

1. Animals deprived of drinking water

a. Six female Sprague-Dawley rats, 4 months old litter mates, were

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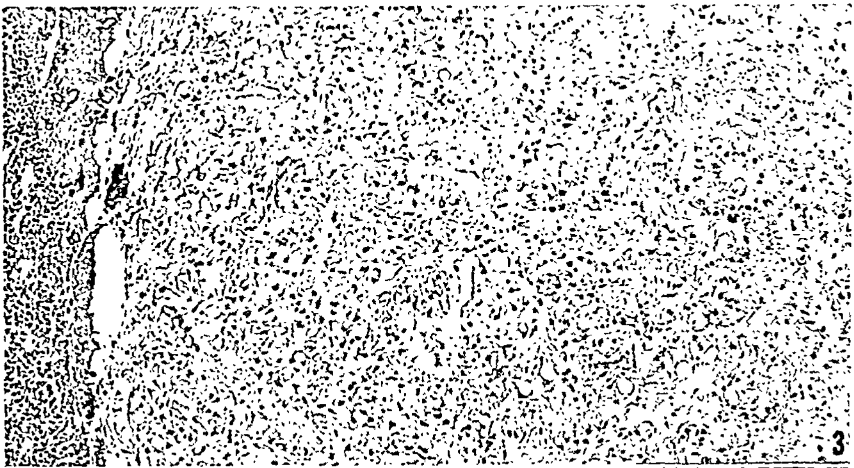
FIG. 3. Pars nervosa of a rat depleted of stainable material after 13 days of ingestion of a 2.5% solution of sodium chloride. Vascular perfusion with Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxyline phloxine, photomicrograph. ×100.

FIG. 4. Re-accumulation of stainable material in the pars nervosa of a rat which was allowed to drink tap water for 2 days following 13 days of dehydration (2.5% NaCl). Technique and magnification as in Figure 3.

FIG. 5. Further re-accumulation of stainable material in the pars nervosa of a rat which was allowed to drink tap water for 4 days following 13 days of dehydration (2.5% NaCl). Technique and magnification as in Figure 3.



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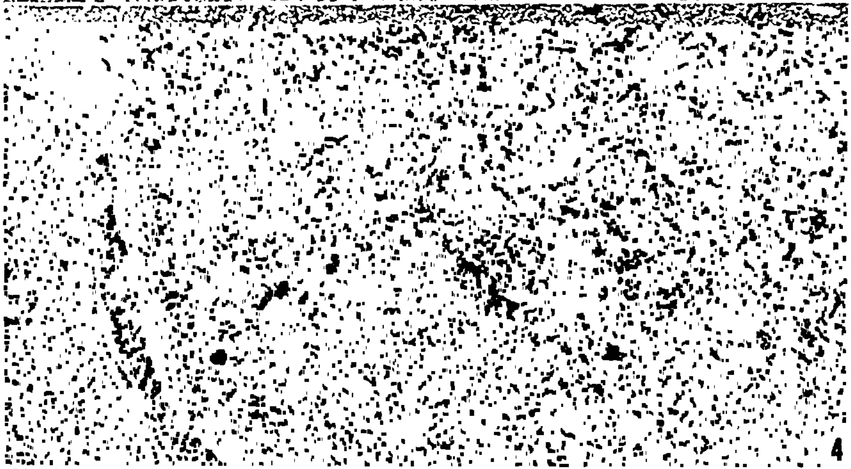

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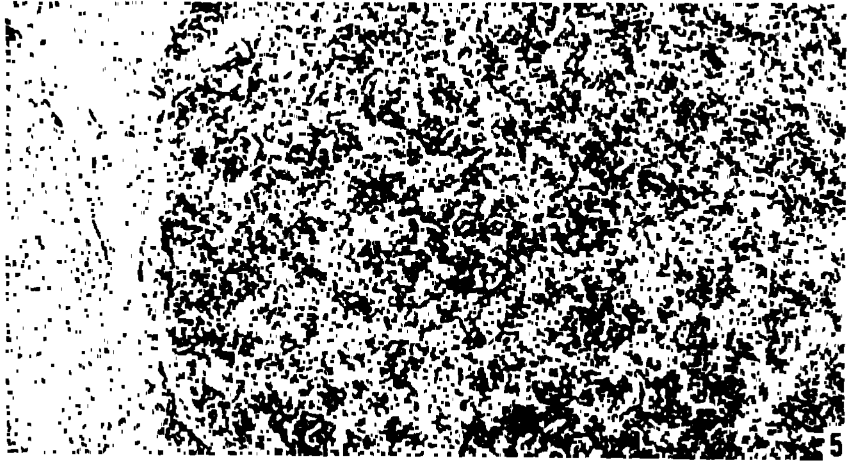

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kept without drinking water for 6 days in July. One animal was sacrificed on the sixth day, the others were given free access to water and were killed 2, 3, 4, 5, and 7 days after the termination of the thirst period.

b. Six female Sprague-Dawley rats of about the same age, but not litter mates, were deprived of water for 13 days in August. Two animals died on the 13th day, a third was killed. The survivors were given water and were then fixed by perfusion with Zenker-formol 1 and 2 days after they had again received drinking water. The sixth rat did not recover and died.

c. Nine female rats of the University of Denver strain which were of about the same age but not litter mates, were deprived of drinking water for 9 days in September. Only three animals survived, of which 2 were killed one and two days after having been given water to drink.

As previously reported (Scharrer, 1952), in none of these animals did the pituicytes in sections stained with the Gomori technique show the faintest granule. The stainable substance did reappear in animals who received drinking water after a period of thirst, but there was no sign that the pituicytes produced it.

We have observed, however, that dehydration produced by the withholding of drinking water is not as suitable a method of depleting the posterior pituitary of its antidiuretic hormone and stainable substance as that produced by the administration of hypertonic saline. During prolonged thirst, the animals refuse to eat and their eyes, nose, and paws become hemorrhagic. There is also much variation in the tolerance of individual animals to the experimental procedure depending upon the moisture content of the air, the climate, and the time of the year at which the animals are subjected to dehydration. Thus, the degree of depletion of the pars nervosa cannot be gaged as accurately in thirsted animals as in those which are given salt solutions of known concentrations. In the latter method of depletion the rats continue to eat, although they may do so to a lesser degree, and they show fewer signs of acute distress during an experimental period of 13 days. Except for very isolated cases, the depletion of the posterior pituitary can be accomplished without the loss of any experimental animals. Consequently, the following sets of experiments were carried out.

2. Animals given a 2.5% solution of sodium chloride instead of drinking water

a. Five young adult rats, litter mates, of the Sprague-Dawley strain were used in order to substantiate further earlier findings which indicated that after 13 days of administration of a 2.5% solution of sodium chloride the depletion of the substance stainable with chrome-alum hematoxylin is usually complete. A study of microscopic sections of the pituitaries of these animals revealed no sign of activity on the part of the pituicytes.[2]

b. Six adult rats, litter mates, of the Sprague-Dawley strain were subjected to saline dehydration for a period of 13 days after which time they were again allowed tap water. One rat was sacrificed on the first day, 2 rats on the second day, 2 on the third day, and 1 on the fourth day following reinstitution of tap water in the diet.

c. Eight adult rats, litter mates, of the Sprague-Dawley strain were subjected to the same experimental procedure as described in 2b. Two rats were perfused with Zenker-formol on the fourth day, 2 on the fifth day, 2 on the sixth day, and 2 on the ninth day following the period of dehydration.

Upon substitution of water for the hypertonic saline a progressive reaccumulation of secretory material was noted in the pars nervosa (Figs. 3 to 5). The first few days following the period of dehydration might be considered crucial with respect to the activity of the pituicytes. At no time were intracellular granules stainable with Gomori's chrome hematoxylin seen in any of these cells (Fig. 6). Rather, the stainable material

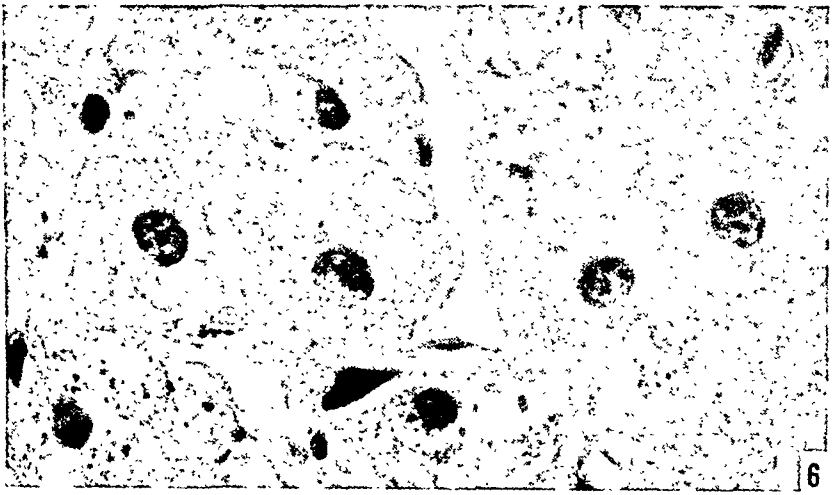

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FIG. 6. Cells of the pars nervosa of a rat which had been kept without drinking water for 9 days. There is no indication that these cells elaborate any material stainable with chrome hematoxylin. Vascular perfusion with Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxylin phloxin. ×1100.

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[2] Chambers (1945), Selye (1950), Ortmann (1951) and Hild (1951 a) have reported the occurrence of numerous mitoses in the neural lobes of dehydrated rats and frogs. The significance of these mitoses is not clear. They do not occur in water starved dogs (Personal communication of Dr. W. Hild).

can first be seen to accumulate in the perivascular areas which Vazquez-Lopez (1942) described as clear spaces and in which the fibers of the hypothalamo-hypophysial tract terminate.

DISCUSSION

The explanation of the mechanisms operating in the clinical entity known as diabetes insipidus proposed by Ranson and his coworkers is based in part on the presumed innervation of pituicytes by nerve fibers derived from hypothalamic nuclei, and in part on the premise that the pituicytes elaborate the pharmaco-dynamic factors obtainable from the neurohypophysis. This interpretation is still held by many workers in the field (Holzer and Hölscher, 1949; Noble, Plunkett and Taylor, 1950; Harris, 1951; Pickford, 1952), although there is ample evidence to the effect that the hormones of the posterior lobe are the products of neurosecretory cells in the hypothalamus. We feel, therefore, that a clarification of the role of the tissue elements in the elaboration of the hormones attributed to this part of the pituitary is of importance.

Ranson and his coworkers believed that the cells producing the antidiuretic hormone could no longer function when deprived of nervous impulses originating in the nucleus supraopticus and probably also in the nucleus paraventricularis. However, Gagel and Mahoney (1936), Mahoney and Sheehan (1936), and Keller (1942) showed that diabetes insipidus does not necessarily result from interruption of the tractus supraoptico-hypophyseus. According to these authors, only lesions in the immediate vicinity of the supraoptic nucleus, cause permanent diuresis; section of the pituitary stalk halfway between hypothalamus and pituitary are not followed by more than a temporary disturbance of the excretion of water. Another aspect concerns the observation of Rasmussen (1938) who pointed out that there are many more fibers in the tractus supraoptico-hypophyseus than would be necessary to innervate the cells of the neurohypophysis. Furthermore, only some of the investigators were able to demonstrate actual contact between nerve fibers and pituicytes (Brooks and Gersh, 1938; Roussy and Mosinger, 1940; Tavares de Sousa, 1943; Romieu and Stahl, 1950). Others state that the pituicytes do not receive nerve terminals from the tractus supraoptico-hypophyseus (Hair, 1938; Rasmussen, 1938; Romeis, 1940; Vazquez-Lopez, 1942; Green 1948). Actually, Vazquez-Lopez (1942), Hagen (1950) and Stotler (1952) have shown that many fibers end at the blood vessels of the posterior pituitary. These fibers do not innervate the vessels, but store neurosecretory material which contains the posterior lobe hormones (Bodian, 1951; Hild, 1952). Consequently, there is little if any basis for the assumption that the pituicytes are stimulated by nervous impulses originating in the nucleus supraopticus or paraventricularis and that such stimulation causes the pituicytes to produce the posterior lobe hormones.

As to the second premise of Ranson, little attention had been paid in the past to the pars nervosa as a possible site of secretory activity, especially since Herring (1908) had presumably established that the hormones found in the posterior pituitary are secreted by cells of the pars intermedia which migrate into the pars nervosa. Cushing (1933, p. 541) emphatically stated, that "it is scarcely conceivable that the neural core of the lobe is capable independently of elaborating a hormone" and subscribed to the theory that the active principles found in the posterior pituitary are derived from the investing pars intermedia. On the other hand, Biggart (1935, 1936) preferred the cells of the pars tuberalis as the site of hormone production. Recently, Büchner (1950) revived the views of Herring and Cushing and combined them with the theory of Ranson, by suggesting that the basophils of the pars intermedia migrate into the neurohypophysis and become subject to the nervous control of the nucleus supraopticus via the tractus supraoptico-hypophyseus. Viewed in this way the function of the posterior lobe of the pituitary becomes indeed "one of the most difficult problems of the biology of the endocrine system" (Büchner, p. 414). It was not until Bucy (1930, 1932) singled out the glia cells in the ox pituitary by giving them a special name ("pituicytes") that the possibility of a secretory activity of the tissue elements of the pars nervosa was given serious consideration. According to Vazquez-Lopez (1942) and Collin and Stutinsky (1949) the term "pituicyte" is, however, not justified. It is only in the ox pituitary which Bucy happened to study, that these cells differ somewhat in their shape from astrocytes or oligodendroglia. In the horse Vazquez-Lopez found no features that would distinguish the glia cells of the neurohypophysis from those of other parts of the central nervous system. The same seems to hold true for the cat and the dog (Sanz Ibáñez, 1934). Spatz (1951) considers the "pituicytes" as incompletely differentiated glia cells. On the other hand, Shanklin (1944) states the following with respect to the pituitary of the pig: "There is a marked similarity between the developing stages of the pituicytes and astrocytes. Each originates from supportive spongioblasts and may pass through bipolar, unipolar and multipolar stages. Like astrocytes they have vascular processes and foot plates. Pituicytes differ from astrocytes, oligodendroglia and microglia for they have many connections between one another whereas the others do not." In the human neurohypophysis various types of pituicytes have been described (Romeis, 1940).

However, the question of the shape of these cells has little if any relationship to the problem under discussion here. Even if a type of glia cell of the kind described by Bucy were to occur generally in the mammalian neurohypophysis, it has never been made clear why a secretory function should be attributed to these cells. The possibility that glia elements in general are potential sources of hormones cannot be excluded (Achúcarro, 1913; Griffith, 1940), but at present no experimental evidence is available in support of such a hypothesis. In the particular case of the cells of the neurohypophysis, our findings in addition to those of Hild, Ortmann and Kratzsch would seem to constitute convincing evidence to show that these cells do not produce the hormones obtainable from the posterior lobe.

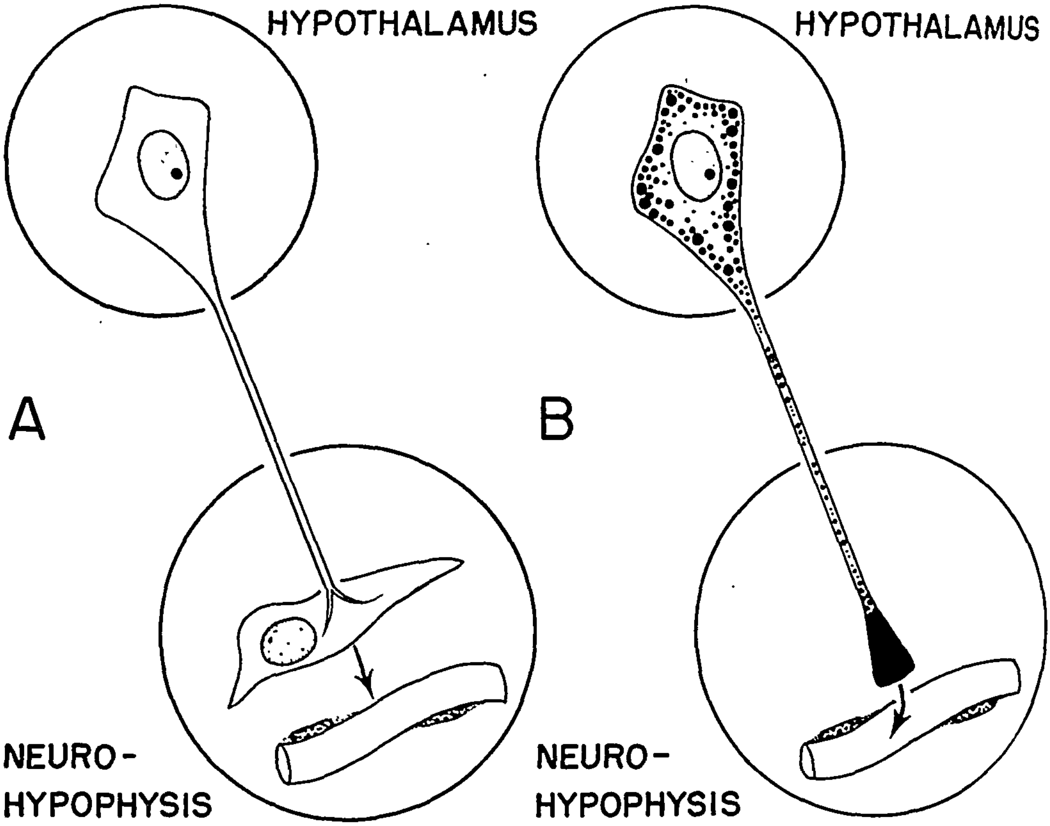

FIG. 7. Diagrammatic representation of two concepts concerning the site of origin and the mode of release of the posterior lobe hormones. A. The currently held view, based mainly on the investigations of Ranson and his coworkers, assumes that cells in the neurohypophysis elaborate and release the posterior lobe hormones under nervous control exerted by hypothalamic nuclei. B. Newer evidence indicates that the posterior lobe hormones are associated with the neurosecretory material produced by nerve cells in the hypothalamus; this material passes along the nerve fibers into the neurohypophysis where it is stored until released into the circulation.

Since, then, it can no longer be maintained that the supraoptic and paraventricular nuclei innervate hormone-producing cells in the neurohypophysis, the theory of Ranson should be replaced by the concept of the neurosecretory origin of the posterior lobe hormones (Fig. 7). According to this concept the nerve cells of the supraoptic and paraventricular nuclei produce the posterior lobe hormones which pass along the fibers of the tractus supraoptico-hypophyseus to attain the neurohypophysis. They are stored there in the nerve terminals[3] from which they are released into the blood vessels.

SUMMARY

The role of the pars nervosa of the pituitary, and more specifically of the pituicytes in the secretion of the antidiuretic hormone, was reexamined.

The antidiuretic hormone occurs in the supraoptic and paraventricular nuclei of the hypothalamus, in the fibers of the tractus supraopticohypophyseus, and in the neurohypophysis in association with material which stains selectively with Gomori's chrome hematoxylin phloxin method (Hild and Zetler, 1951 a,b; 1952 a,b; 1953 a,b). If the pituicytes play a role in the elaboration of the antidiuretic hormone, they should be active in secreting this stainable material in animals which had been dehydrated both by the withholding of drinking water and by the administration of sodium chloride of known concentrations. It was found, however, that the pituicytes have no part in the elaboration of the stainable material and consequently of the antidiuretic hormone.

A critical analysis of the evidence on which Ranson's theory of the function of the posterior pituitary was based, leads one to the conclusion that this theory should be abandoned in favor of the concept of the neurosecretory origin of the antidiuretic hormone.

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[3] The possibility that the posterior lobe hormones are produced by the nerve endings in the neurohypophysis was first suggested by Rasmussen (1938), later discussed by O'Connor (1947) and recently again proposed by Bodian (1951). However, all the available evidence indicates that the hormones are merely stored, but not elaborated in the nerve terminals.

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THE *IN VIVO* METABOLISM OF CORTISONE[1]

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THE application of paper chromatography and infra-red spectrometry has permitted the isolation and identification of the major C-21 and C-19 metabolites excreted in urine, following the administration of adrenosterone (Savard *et al.*, 1953) hydrocortisone and 21-desoxycortisone (Burstein *et al.*, 1953). It has been shown that the C-21 steroids are reduced in the human body to the pregnane (5β) and transformed to the corresponding C-19 etiocholane (5β) derivatives. On the other hand, the Δ^4-3 keto system in adrenosterone is converted predominantly into derivatives of the androstane (5α) series, which indicates that the ethyl side chain in position 17 of these steroids appears to direct the reduction of the $\Delta^{4,5}$ double bond to the 5β form *in vivo.*

The *in vivo* metabolism of cortisone has been described by Dobriner *et al.* (1950, 1951) and Lieberman *et al.* (1951). However, no details were given as to the relative quantities of the metabolites excreted.

In view of the importance of cortisone in endocrinological research and in therapeutics, the study of the metabolism of orally administered cortisone acetate to a normal man has been undertaken. The present paper demonstrates the conversion of cortisone: 1) to pregnane-3α,17α,21-triol-11,20-dione and pregnane-3α,11β,17α,21-tetrol-20-one; 2) to hydrocortisone, and 3) to 17-ketosteroids of the etiocholane (5β) configuration bearing an oxygen function at carbon 11.

METHODS

A 26 year old male patient who had been successfully treated for hepatitis 4 months previously was used in this study when his clinical picture was normal.[2] An oral dose of 500 mg. of cortisone acetate[3] was administered daily for 5 days.

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[2] We are indebted to Dr. G. W. Thorn for the clinical aspects of this study.

[3] The cortisone acetate was generously supplied to Dr. G. W. Thorn for this study by Merck & Company, Rahway, New Jersey.

Steroid isolations were performed on a urine sample which represented 1.8 gm. of the administered cortisone acetate. A 24-hour urine collection just prior to the cortisone treatment served as a control. The steroid conjugates were hydrolyzed, and the ketonic extracts chromatographed on paper in the manner previously described (Burstein *et al.*, 1953). The isolated steroids were identified by chromatographic mobility on paper, melting points, and infra-red analyses. We are indebted to Dr. Harris Rosenkrantz for the valuable interpretation of the infra-red spectra. The melting points were determined by the Kofler Micro Hot Stage melting point apparatus, and are recorded uncorrected.

EXPERIMENTAL AND RESULTS

Control Urine: The corticosteroid fraction (which stayed at the starting line following chromatography in the toluene-propylene glycol (T.-P.G.) system for 4 hours) of the ketonic material of the 24 hour control urine contained 1.2 mg. of formaldehydogenic steroid. The quantities of the 17-ketosteroids contained in the zones having the mobility of the order of 11β-hydroxyandrosterone (0.6 mg.) and etiocholane-3α-ol-11,17-dione (0.5 mg.) were insufficient for crystallization and infra-red spectrometry. No etiocholane-3α,11β-diol-17-one was detected. Etiocholane-3α-ol-17-one (5.2 mg.) and androsterone (3 mg.) were isolated in crude form and identified by their infra-red spectra.

Cortisone administration urine: The results obtained with the cortisone urine are listed in Table 1. In the ring A saturated corticosteroid fraction, which was rechromatographed in a chloroform-formamide system (Schindler and Reichstein, 1951), pregnane-3α,11β,17α,21-tetrol-20-one and pregnane-3α,17α;21-triol-11,20-dione were obtained. The latter steroid was crystallized directly but the former steroid was acetylated and rechromatographed in the ligroin-propylene glycol (L.-P.G.) system (52 hours).

TABLE 1. ISOLATION OF STEROIDS FROM CORTISONE URINE

Chrom. zone	System	Hrs.	Location of zone: dist. from starting line cm.	Weight of isolated steroid		Compound	Identifying characteristics
				Crude, mg.	Crystal, mg.		
1-I	Ch.-F.*	12	5.5– 8.5	4.6		Pregnane-3α,11β,17α,21-tetrol-20-one	Mobility§
1-I acet-	L.-P.G.†	52	3.2– 5.8	2.0	1.0	Pregnane-3α,11β,17α,21-tetrol-20-one-3α,21-diacetate	M.P. 208–215°; Mobility; Infra-red spectrum
1-II	Ch.-F.	12	12.5–17.2	44	18	Pregnane-3α,17α,21-triol-11,20-dione	M.P. 194–198°; Infra-red spectrum
1-III	T.-P.G.‡	72	5.0– 8.1	5		Hydrocortisone	Mobility; Infra-red spectrum
1-IV	T.-P.G.	72	9.0–12.0	1.1		Unknown	
1-V	T.-P.G.	72	13.2–16.0	2.1		Cortisone	
1-VI	T.-P.G.	72	23.0–27.0	0.7		Unknown	
2-I	L.-P.G.	96	1.5– 3.0	11.3	4.3	Etiocholane-3α,11β-diol-17-one	M.P. 232–36°; Infra-red spectrum
2-II	L.-P.G.	72	6.0–12.0	9.0	3.2	Etiocholane-3α,ol-11,17-dione	M.P. 180–82°; Infra-red spectrum
3-I	L.-P.G.	24	12.5–18.0	6.3		Δ^9-Etiocholane-3α-ol-17-one	Mobility; Infra-red spectrum
3-II	L.-P.G.	24	23.7–29.0	4.4		Δ^9-Androstene-3α-ol-17-one	Mobility; Infra-red spectrum

* Chloroform-Formamide.
† Ligroin-Propylene-Glycol.
‡ Toluene-Propylene-Glycol.
§ Possessing the mobility of the order of the respective compound.

Both pregnane compounds were identified by their infra-red spectra. In the Δ^4-3-keto-corticosteroid fraction, hydrocortisone and cortisone were found and identified by their chromatographic mobility on paper and infra-red spectra. The hydrocortisone gave a strong greenish fluorescence in sulfuric acid and no fluorescence was obtained with the isolated cortisone. Two other blue tetrazolium reducing compounds were observed on the chromatogram. One was located between the hydrocortisone and cortisone zones (Zone 1-IV) and the second occurred immediately ahead of the cortisone zone (Zone 1-VI). The quantity of these substances was insufficient for further identification.

Among the 17-ketosteroids, etiocholane-3α,11β-diol-17-one (from Zone 2-I), etiocholane-3α-ol-11,17-dione (from Zone 2-II), Δ^9-etiocholene-3α-ol-17-one (from Zone 3-I) and Δ^9-androsten-3α-ol-17-one (from Zone 3-II) were isolated and identified by their mobilities, melting points and infra-red spectra (Table 1). Neither etiocholane-3α-ol-17-one nor androsterone were found in the cortisone urine, although significant quantities of these steroids were excreted during the control period (Table 2).

TABLE 2. STEROID ISOLATION IN CORTISONE METABOLISM STUDY

Steroid	Quantity isolated (crude) mg.	
	Control[1] urine	Cortisone urine
Pregnane-3α,11β,17α,20-tetrol-20-one-3α,21-diacetate	6.0 mg.[2]	2.0 (0.11%)[3]
Pregnane-3α,17α,21-triol-11,20-dione		44 (2.5%)
Hydrocortisone		5 (0.28%)
Cortisone		2.1 (0.11%)
Etiocholane-3α,11β-diol-17-one & Δ^9-Etiocholene-3α-ol-17-one	absent	17.6 (0.98%)
11β-Hydroxyandrosterone and Δ^9-Androsten-3α-ol-17-one	3.0	4.4 (0.24%)
Etiocholane-3α-ol-11, 17-dione	2.5	9.0 (0.5%)
Etiocholane-3α-ol-17-one	26.0	absent
Androsterone	15.0	absent

[1] Values given are corrected to the equivalent of a urine collection of the cortisone administration period; calculations are based on the maximum amounts of crude material found.

[2] This value was determined by the formaldehydogenic method on the corticosteroid fraction.

[3] The percentages in parentheses are the conversion estimates of the administered cortisone equivalent to the urine available in this study (1.8 gm.).

DISCUSSION

The steroids isolated from both control and cortisone administration urines are listed in Table 2.

Similar to the results obtained in the hydrocortisone study (Burstein *et al.*, 1953), pregnane-3α,17α,21-triol-11,20-dione constituted the major steroid found in the corticosteroid fraction. Only a small quantity of pregnane-3α,11β,17α,21-tetrol-20-one was obtained. The conversion of cortisone to hydrocortisone occurred to the extent of 0.28 per cent. In a similar study Mason (1950) has indicated that hydrocortisone may be present following cortisone administration. The quantity of the excreted cortisone in the present study is of the same order of magnitude as that of the excreted hydrocortisone. This finding is similar to that which has been obtained following the administration of hydrocortisone (Burstein *et al.*, 1953). The pattern of the urinary corticosteroids excreted is the same whether cortisone or hydrocortisone is orally administered. The conversion of cortisone to etiocholane-3α, 11β-diol-17-one (the major 17-ketosteroid) and etiocholane-3α-ol-11,17-dione occurs to a lesser degree than the corresponding conversions following hydrocortisone administration (Burstein *et al.*, 1953). On the other hand, the extent of the conversion of cortisone to the pregnane derivatives appears to be larger than the corresponding conversion of hydrocortisone to these metabolites. During the cortisone administration period only a slight increase of 11β-hydroxyandrosterone and its dehydration product Δ^9-androstene-3α-ol-17-one was found. The difference between control and experimental urines was so slight that it is questionable whether cortisone is actually degraded in the human body to this 17-ketosteroid.

Although etiocholane-3α-ol-17-one and androsterone were isolated from the control urine, these steroids were not found in the cortisone urine. This finding is in accord with our studies on the influence on the excretion of these two 17-ketosteroids of high dosages of hydrocortisone (Burstein *et al.*, 1953).

Pregnane-3α,17α-diol-11,20-dione, which was isolated following cortisone and ACTH treatment by Lieberman *et al.* (1951) was not found in the present study.

SUMMARY

1. A total of 2.5 gm. of cortisone acetate has been administered to a man.

2. The following metabolites have been isolated and identified: pregnane-3α,11β,17α,21-tetrol-20-one as the diacetate, pregnane-3α,17α,21-triol-11,20-dione, hydrocortisone, cortisone, etiocholane-3α,11β-diol-17-one, Δ^9-etiocholene-3α-ol-17-one and etiocholane-3α-ol-11,17-dione.

3. No definitive evidence could be found for the conversion of cortisone to 11β-hydroxyandrosterone.

4. Etiocholane-3α-ol-17-one and androsterone which were present in the control urine in significant quantities, were completely absent from the urine following cortisone administration.

ADDENDUM

In a recent publication by Burton, Keutmann, Waterhouse (*J. Clin. Endocrinol. & Metabolism* **13**: 48. 1953), cortisone, hydrocortisone, pregnane-3α,17α,21-triol-11,20-dione and possibly pregnane-3α,11β,17α,21-tetrol-20-one were identified by paper chromatographic methods, following the parenteral administration of cortisone.

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THE EFFECT OF ADRENALECTOMY, SURGICAL TRAUMA, AND ETHER ANESTHESIA UPON THE CAPILLARY RESISTANCE OF THE ALBINO RAT*

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CAPILLARY leakage associated with widespread vascular dilatation has long been considered as a typical change after adrenalectomy. The regular finding of hemoconcentration (Athanasiu and Gradinesco, 1909) and of increased protein in the lymph (Cope, Brenizer and Polderman, 1942), and furthermore the observation that spreading of intradermally injected India ink is augmented after adrenalectomy in the mouse (Opsahl, 1949), have been regarded as evidence for the increased capillary *permeability*. That adrenalectomy may affect the capillary *resistance* as well was shown recently by Eichholz, Staab and Taugner (1951). Studying the blood pressure and the capillary resistance of rats during the first two weeks following adrenalectomy these authors found a decrease of the resistance from 76 to 46 cm. Hg as early as the third day. It is of interest that the change in the capillary resistance preceded the fall of the blood pressure by 1–2 days, and that both, hypotension and decreased resistance, could promptly be restored by administration of saline and Percorten (desoxycorticosterone glucoside in aqueous suspension).

While total removal of the adrenal glands followed by administration of cortical extract or isolated hormones may give information concerning the role of this organ in the maintenance of the capillary integrity, it is likewise possible that any change that may be found following this procedure may be due not only to the absence of the adrenal glands but also to the surgical trauma itself or even to the anesthetic. For this reason distinction should be made between early and late postoperative periods, the borderline between them being the time when the effects of the nonspecific factors become minimal or absent. In the early period, in addition, there is a fundamental disturbance in all functions of the organism with considerable vascular dilatation and capillary stasis but—in the rat provided with an increased salt supplement—compensatory readjustment soon occurs. This late postoperative state is more suitable for a study of

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any partial function of the body as well as for the testing cortical extracts or corticoids than is the early period.

The present work on the effect of adrenalectomy upon the capillary resistance was therefore preceded by a study of the non-specific effect of surgical trauma and anesthesia. The effect of adrenalectomy itself was studied during a period of 2–8 months.

METHOD AND PROCEDURE

Mature albino rats of the Sprague-Dawley strain and of both sexes with an average weight of 250 gm. and fed Purina Dog Chow were used. Twenty-four animals were adrenalectomized, 16 were subjected to sham adrenalectomy and 16 to ether anesthesia without surgical trauma. Deep ether anesthesia was given for 18 minutes. The adrenalectomy as well as the sham operation took 12 minutes. The length of incisions through skin and dorsal musculature as well as the number of stitches applied were standardized. In order to render the non-specific effects conspicuous these procedures were deliberately made in a more protracted and circumstantial manner. In the sham operation the adrenals were exposed—but not touched. Adrenalectomized rats were given 1% NaCl as drinking water. In addition 3 groups of rats, each consisting of 9 animals, were subjected to the same procedures (ether anesthesia, sham operation, adrenalectomy) with the purpose of studying the effect of ACTH and the corticoids. The animals receiving only ether anesthesia and those with sham operation were primed with the hormones before the respective intervention and for a certain period following it. The adrenalectomized rats were treated with the hormones in the late postoperative state.

The device used in these experiments for testing the capillary resistance consisted of a vacuum jar which was connected to an electrically powered suction pump on one direction and to a small plastic bell (of an inner diameter of 7 mm.) with a stopcock in the other direction. Degree of suction was measured by an intercalated mercury manometer. The skin of the abdomen of the animals was prepared by an electric hair clipper but it was not shaved. Shaving, in our experience, may irritate the skin and give rise to erroneous readings. A thin layer of liquid petrolatum applied to the skin before the testing provided a good surface for the suction cup to cling to and also facilitated viewing of the small petechiae. Suction was employed for 60 seconds. The least negative pressure given in cm. Hg. still capable of producing hemorrhage was considered as the value of the capillary resistance. As it was found that spasm of the precapillaries may lead to falsely high readings and thus interfere with the accuracy of the test, much care was taken to avoid or at least to recognize this condition. For this reason holding the animal in the palm of the glove hand was preferred, whenever possible, to restraining it by tieing it down to the board.

About one hundred healthy, untreated animals at different age levels have been tested for a period of over one year in order to determine the normal variation of the capillary resistance. It was found to range rather widely. The resistance was considered highest when no bleeding occurred even with a suction of 70 cm. (the average barometer reading in Omaha is approximately 730 mm.). It was then marked >70 cm. Healthy, well developed immature animals may show such a high resistance, the values being above 60 cm. Fully developed rats, sometimes all members of the same litter, may disclose a lower resistance (between 40 and 60 cm.), and apparently healthy animals may occasionally be found with exceptionally low levels (35–39 cm.). Though each animal tends to maintain its individual level during several months of observation with a fluctuation not exceeding ±5 cm., a general tendency to a gradual decrease with advancing age is noticeable. A capillary resistance under 27 cm. is considered to be definitely pathologic. Values of 35–27 cm. are looked upon as borderline. Thus the following arbitrary classification of the capillary resistance was found useful.

		Hg cm.
Normal	High	>70–61
	Medium	60–46
	Low	45–35
Intermediate		34–27
Pathologic	Moderately decreased	26–11
	Very low	10–0

The wide range of normalcy appeared to us rather enigmatic at first; its meaning, however, soon became evident in the course of this study.

RESULTS

Figure 1 shows typical patterns of capillary resistance change following the three types of intervention.

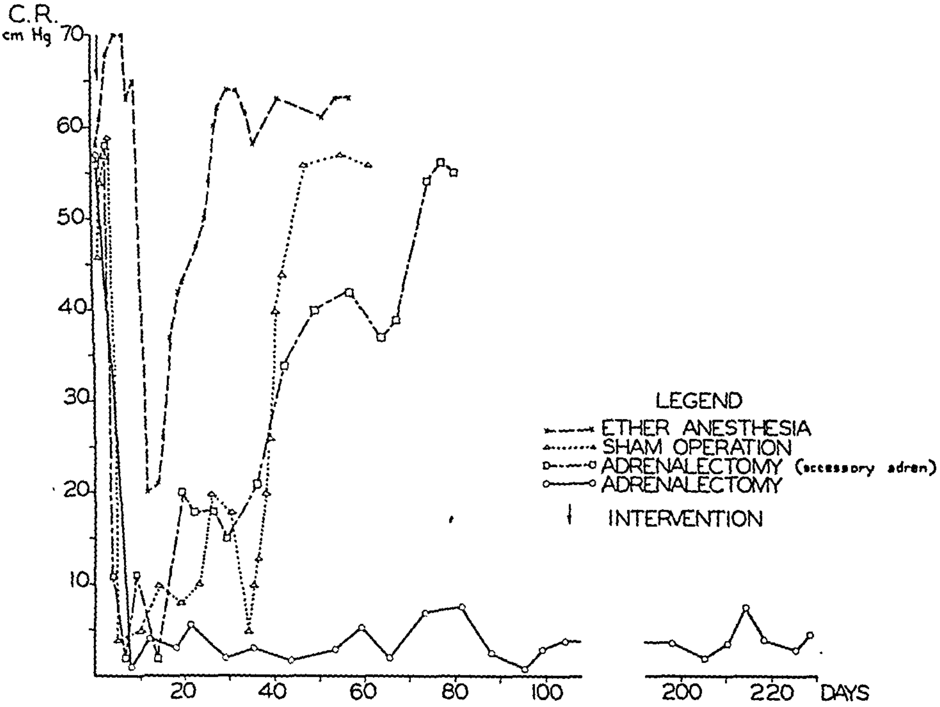

FIG. 1. Typical change in the capillary resistance of the rat following ether anesthesia, surgical trauma (sham adrenalectomy) and adrenalectomy. Change in the capillary resistance after adrenalectomy in a rat with accessory adrenal tissue shows no significant difference as compared to sham adrenalectomy.

Ether anesthesia. There is no change during the first 3–4 days although a tendency for the resistance to increase may often be noticeable. Between the fourth and the 12th day a more or less rapid decrease ensues and the resistance remains at pathologic levels for 1 to 6 days, whereafter it gradually rises and finally returns to its original value. The entire phenomenon, which may be termed "capillary crisis," occupies not more than 2 weeks. No other pathologic signs were observed during this entire period of capillary derangement. Wide individual variations were noted in onset, degree and duration of this "crisis." In three out of 16 animals the capillary resistance did not fall lower than the intermediate zone and in two strong male rats the crisis was entirely missed.

Surgical trauma (sham adrenalectomy). Here the typical crisis is more conspicuous than in the previous (ether control) experiments. Without exception the resistance began to decrease after the fourth day and then dropped in a rather abrupt manner and remained at pathologically low levels for a longer period of time than in the ether controls (14 days on the average). Recuperation was likewise more prolonged. The entire cycle took about one month. The critical drop of the resistance was sometimes preceded by a transient rise. The animals responded in a more uniform and marked way than when anesthesia was given without surgical trauma, although considerable individual variation in the capacity of restoring the resistance was commonly noted. During the first few postoperative days the animals were rather lively. Many of them, though not all, showed signs of general malaise and weakness during the second and third postoperative weeks. They did not eat well, became careless about their fur and their weight curves flattened. Some of them developed scattered small eczematous plaques.

Adrenalectomy. Being thus familiar with the response of the rats to anesthesia and surgical trauma, the effect of adrenalectomy was next studied. Twenty-four animals were adrenalectomized in deep ether anesthesia using the standardized technic as employed in the sham operation. Four of them died in the first days following operation, 10 died within two months, 9 survived for more than three months. In two of the 24 rats it was found that removal of the adrenals was not complete, in two other rats accessory adrenal tissue was revealed. The curve of the capillary resistance of these latter four animals was essentially the same as that seen after sham operation, though the restoration was more protracted. Figure 1 represents the behavior of the capillary resitance of one of the two animals with accessory tissue. The remaining 16 rats (the 4 that died within the first postoperative days are not considered here) showed a uniform pattern that was entirely different and is believed to be characteristic of the absence of the adrenals (Fig. 1.) The resistance decreased precipitously after the second day following removal of the glands and became, without exception, definitely pathologic after the 5th day in contrast to the more protracted slope in the curves shown by some of the sham operated animals. The essential difference, however, lies in the fact that whereas after sham operation the resistance returns to previous levels, it remained permanently low after adrenalectomy. The capillary resistance of several animals has been followed for 5 to 9 months after adrenalectomy and found to be consistently at or below 10 cm. Hg.

The effect of ACTH and Corticoids upon the pathologically low capillary resistance following adrenalectomy. The late postoperative period (2–8 months after operation), in which the animals are in a relatively balanced condition, having permanently low capillary resistance, seemed to be very suitable for checking hormonal effects. Figure 2 leaves no doubt about the effectiveness of cortisone.[1] In contrast, DOCA in high doses administered in aqueous[2] or oily[3] medium subcutaneously or intramuscularly was entirely ineffective. The slight rise in the curve occurring in some animals was

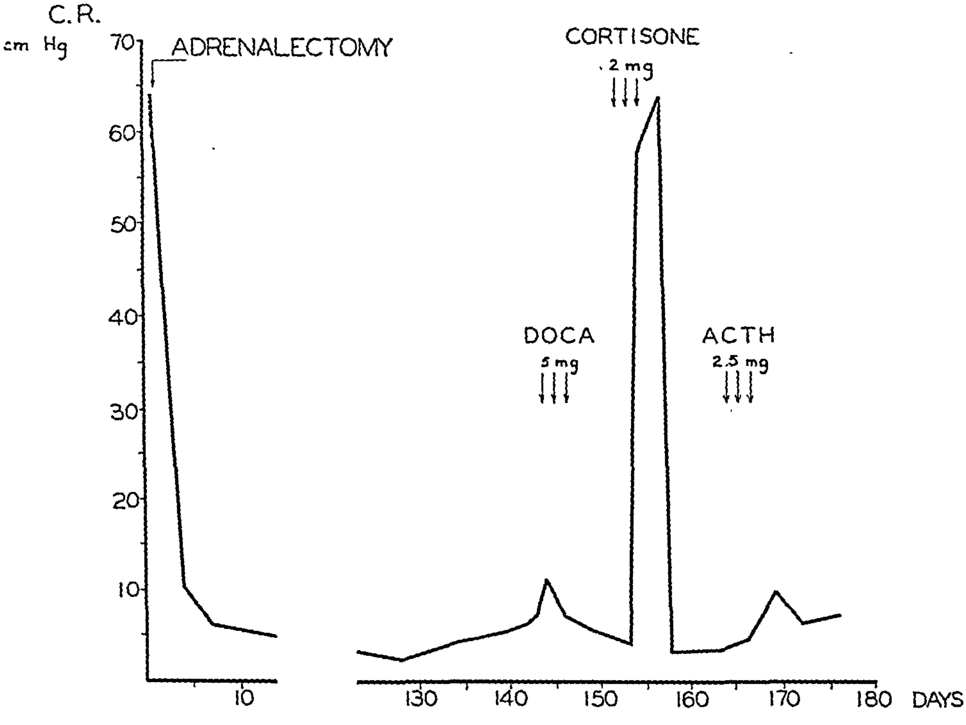

FIG. 2. Effect of cortisone, DOCA and ACTH on the capillary resistance of adrenalectomized rat in the late postoperative period.

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[1] 11-dehydro-17 hydroxycorticosterone 21-acetate (Cortone acetate, Merck).

[2] Desoxycorticosterone acetate aqueous macrosuspension (Bell Pharm. Co.).

[3] Desoxycorticosterone acetate in sesame oil (Cortate, Schering).

due to an edema of the skin caused by the huge doses of DOCA. ACTH[4] likewise showed no effect.

The effect of ACTH and Corticoids upon the capillary crisis elicited by surgical trauma and by ether anesthesia on normal (non-adrenalectomized) animals. Figures 3 and 4 show clearly evidence that cortisone is able to prevent the capillary crisis occuring after surgical trauma or ether anesthesia. All treated animals showed high normal resistance during the entire period of two weeks during which the capillary crisis might have been expected. In contrast all the animals primed with ACTH or DOCA disclosed the usual capillary response, i.e., these substances were unable to prevent the crisis. When, however, cortisone was withdrawn from the sham operated animals at a time when the resistance of the matched animals treated with ACTH or DOCA already returned to normal—a typical crisis set in. In case of anesthesia alone the resistance remained within normal values

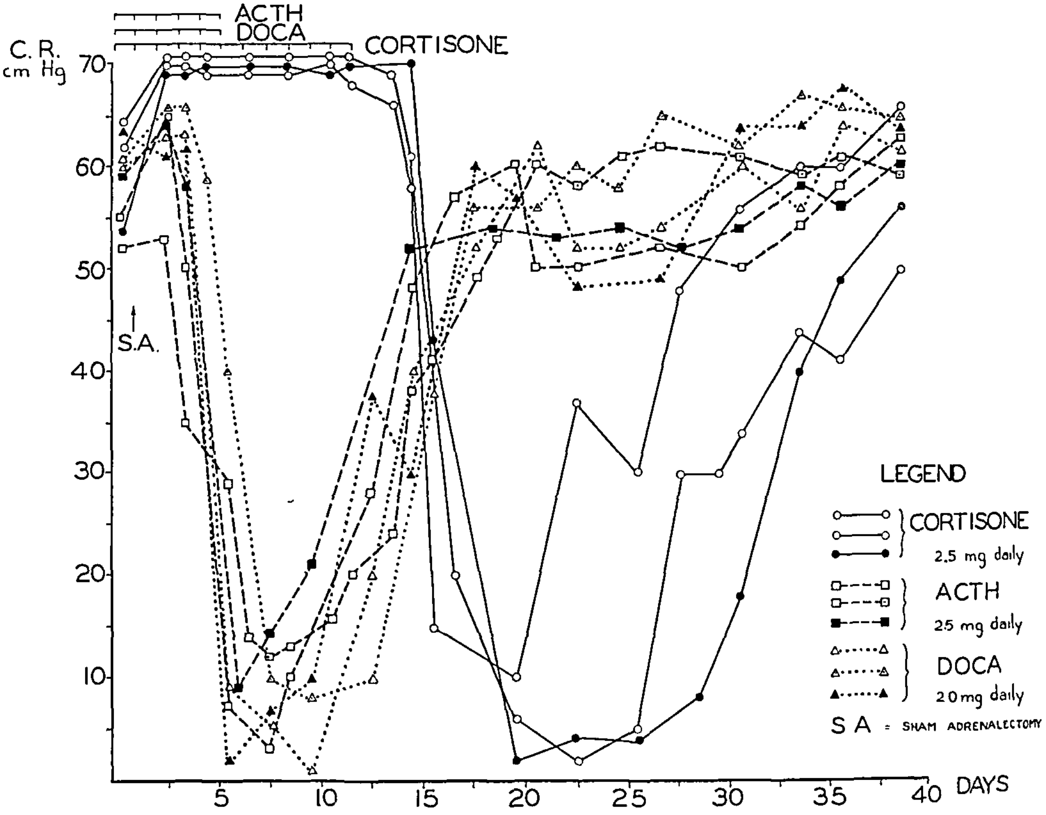

FIG. 3. Effect of cortisone, DOCA and ACTH on the "capillary crisis" due to surgical trauma. DOCA and ACTH were unable to prevent the crisis while cortisone was fully effective. Withdrawal was followed by a typical "secondary capillary crisis."

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[4] Lyophilized adrenocorticotropic hormone (ACTHAR, Armour Lab.).

after cortisone was discontinued. However, there was a small indentation in the curve suggesting a rudimentary form of a crisis.

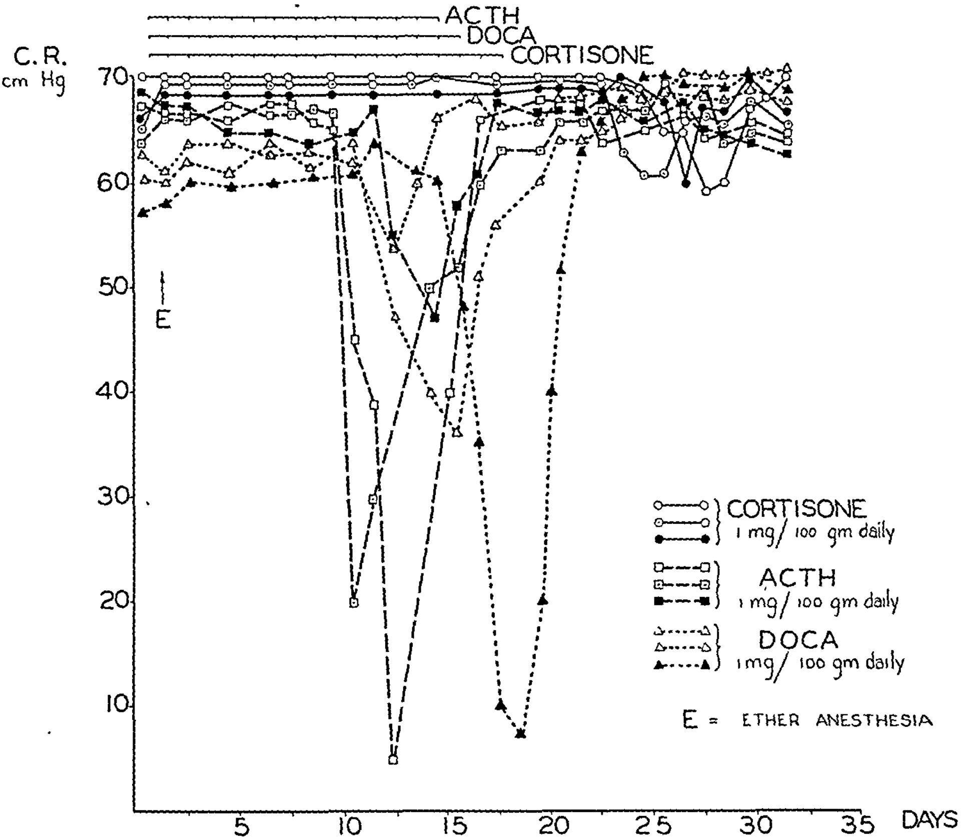

FIG. 4. Effect of cortisone, DOCA and ACTH on the capillary crisis elicited by ether anesthesia. In these experiments the rats were kept in an atmosphere containing 6 vol. % ether for 90 minutes. Cortisone was fully effective in preventing the capillary crisis whereas DOCA and ACTH failed to do so. Rudimentary form of "secondary capillary crisis" after withdrawal of cortisone.

DISCUSSION

The behavior of the capillary resistance after adrenalectomy studied in a long range experiment of several months' duration definitely suggests that the maintenance of the normal capillary resistance is a function of the adrenal cortex. Administration of ACTH, cortisone, and DOCA in the late postoperative phase seem to indicate that cortisone is involved in this function. Eichholz *et al.* (1951), as mentioned above, attribute the same role to DOCA.

The present study had already been completed when the paper of Eichholz *et al.* became available to us. Thus without repeating their experiments it can only be stated that DOCA was found ineffective under our experimental conditons. It is presumed that the explanation for this discrepancy may be found among the following factors.

	Eichholz et al.	*Authors*
Observation made in period	early postoperative	late postoperative
Duration of observation	two weeks	up to 8 months
Range of capillary resistance tested	76–46 cm. Hg (Within Normal zone)	70–0 cm. Hg (including Intermediate and Pathologic zones
Administration of NaCl	parenterally (mostly) with DOCA whenever it was tested	Constant oral supply during entire period of observation
Preparation used	Percorten, aqueous (desoxycorticosterone glucoside)	Desoxycorticosterone acetate aqueous macrosuspension (Bell Pharm Co.); In sesame oil (Cortate, Schering)

Since the early observation of Leriche upon the "maladie post-opératoire" and its recognition as a pathologic entity (an example of the alarm reaction by Selye) the systemic changes caused by traumatic injury have been the subject of considerable interest and innumerable studies. Nevertheless little is known about the behavior of the capillaries in this condition and the available data are, in addition, rather conflicting. On the one side there stands the still debated question whether or not a traumatic shock may produce capillary *leakage* at a distance from the site of the injury (pro: Selye, contra: McMichael, 1945; Nobel and Gregersen, 1946)—on the other side the observation of Cole, Shaw and Fraser (1950) suggesting that trauma may lead to a decrease of the permeability. Concerning capillary resistance only two data are available (Ungar, 1944; Robson *et al.*, 1950). both indicating an *increase* after tissue damage. No reference whatsoever could be found in this relation concerning ether anesthesia.

In view of these data in the literature the capillary crisis found in our experiments after relatively minor injuries such as sham adrenalectomy or anesthesia seemed rather enigmatic at first glance. When one compares these curves, however, with those of the adrenalectomized animals and considers the effect of cortisone in all these conditions, these findings become more comprehensible.

If the adrenal cortex governs the capillary resistance and if cortisone is a mediator of this function, the critical drop of the capillary resistance following stress and the period of low resistance that ensues thereafter must necessarily mean either that the stimulating effect of the pituitary upon the adrenal was lacking during this time or that the adrenals were *not* responsive. As ACTH failed to prevent the capillary crisis (note: The animals were not adrenalectomized) whereas cortisone was fully effective the second possibility must be thought of. Since it is known that the demand of the organism for corticoids is increased during stress the explanation may be offered that this unresponsiveness of the adrenal and, hand in hand with it, the capillary crisis, are expressions of a transient hypofunction or depletion. (The term "exhaustion" is deliberately avoided to prevent confusion with Selye's concept of "state of exhaustion.")

Details of an analysis of this remarkable condition will soon be published on the basis of studies that have been made with various other forms of stress. The phenomenon of the "secondary capillary crisis" occurring after discontinuance of cortisone on animals in which the primary crisis due to a stress was prevented by it will also be the subject of a special study.

SUMMARY

The change of the capillary resistance of the albino rat following adrenalectomy was studied for a period of 2 to 8 months. In order to rule out the possible effects of surgical trauma and anesthesia (factors necessarily involved with the adrenalectomy) upon the capillary resistance, some groups of rats were sham operated in ether anesthesia or subjected to the anesthesia alone.

After *sham adrenalectomy* the capillary resistance changed in a typical way showing a pattern with 4 successive phases: (1) a more or less pronounced increase in the first days; (2) a critical drop at the end of the first or in the course of the second week; (3) a state of pathologically low resistance lasting about two weeks; (4) a period of recuperation. The entire phenomenon—it may be termed "capillary crisis"—takes about one month. Though individual variations were often noticed, on the whole, the response of the animals was remarkably uniform.

Following *ether anesthesia* the same pattern was observed, mostly in somewhat telescoped and less conspicuous form. Individual variations were more common.

After *adrenalectomy* only the second and third phases of the above pattern were seen, i.e., the initial rise was absent and the capillary resistance remained at low levels during the entire period of observation (up to 9 months). In the presence of accessory adrenal tissue the animals responded in the same way as after sham operation, although restoration was more protracted.

Since the first phase of the capillary crisis (initial rise of the resistance before its critical drop) in the sham operated or merely anesthetized animals may often be absent or easily missed, it is obvious that during the first 3 to 4 weeks following operation there is no significant difference between adrenalectomized and sham operated animals as far as capillary

resistance is concerned. In view of this observation as well as of the possibility that hemodynamic changes ensuing during the first weeks after removal of the gland may also influence the capillary integrity, it must be inferred that the late postoperative period is, in fact, more suitable that the early period for a study of the effect of adrenalectomy upon the capillary resistance as well as for testing hormonal or other preparations.

A study in this late postoperative period showed that cortisone is able to restore the capillary resistance promptly. ACTH and DOCA (both aqueous and oily) were found ineffective. Cortisone given to sham operated or merely anesthetized animals before the procedures prevented the capillary crisis. DOCA and ACTH failed to do so.

These findings suggest that the adrenal cortex is intimately connected with the resistance of the capillaries and that cortisone seems to be involved in this function.

The explanation is offered that the "capillary crisis" found after surgical trauma or anesthesia may be an expression of a transient hypofunction or depletion of the adrenal cortex.

Acknowledgment

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THE EFFECTS OF ANDROGEN AND ESTROGEN IN CASTRATED AND HYPOPHYSECTOMIZED C_3H MICE

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INTRODUCTION

ESTROGEN-ANDROGEN antagonism may be defined as the suppression or modification of the effect of one hormone on an end-organ or tissue by the opposite sex hormone. The phenomenon has been demonstrated in many experiments (review, Burrows, 1949), but it is not clear to what extent antagonism occurs at the site of action on the end-organ, or to what extent it is brought about indirectly through changes in pituitary or gonadal function. The experiments of Moore and Price (1932) seemed to indicate that no estrogen-androgen antagonism existed except as mediated through the influence of the gonadal hormones on pituitary gonadotropic function. Martin (1951), on the other hand, has stated that estrogen-androgen antagonism probably holds for any tissue affected by either hormone, and that inhibitions are due to direct antagonistic action in the tissues.

The present study was designed to indicate the extent of estrogen-androgen antagonism occurring in animals from which pituitary and gonadal influences were eliminated, at a constant dose level of the two sex hormones.

METHODS

A total of 160 C_3H strain mice were used in the experiment. The animals were singly housed, and water and Purina Fox Chow were provided ad libitum. Hypophysectomy by the method of Thomas (1938), and castration were performed in 140 of the mice. Completeness of hypophysectomy was evidenced by a continuing loss of weight in every animal used in the experiment, absence of visible pituitary tissue on examination with a dissection microscope at post-mortem, and absence of recognizable pituitary tissue in serial sections of the decalcified sella turcica in 4 representative animals.

Injected hormones included α-estradiol, diethylstilbestrol, and testosterone propionate, hereafter referred to as estradiol, stilbestrol, and testosterone, respectively. The total daily hormone dose was injected subcutaneously in 0.03 ml. of oil, in one of 3 locations varied in regular rotation.

The mice were 3 to 4 months old at operation. Injections were begun 31 to 35 days after operation, and continued daily for 14 days. Vaginal smears were taken daily by lavage in all females during the injection period. The mice were killed with ether on the

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day following the last injection. Organ and body weights were obtained from each animal, and tissue sections and mammary whole mounts were prepared from 2 or more animals in each group.

RESULTS

Preliminary experiment to determine minimum doses of hormones producing maximal effect in castrated and hypophysectomized mice.

It is well known that the relative dosage of androgen and estrogen may determine whether antagonistic, cooperative, or independent effects will be produced in a given tissue (Burrows, 1949). Selection of doses for the present experiment was made as follows. Weights of seminal vesicles with their retained secretion in 6 castrated and hypophysectomized male mice, subjected to graded doses of testosterone injected daily for 14 days, indicated that the minimum dose producing a maximum increase was about 20 μg. daily. Eight castrated and hypophysectomized female mice were injected with graded doses of stilbestrol, and six were injected with graded doses of estradiol, daily for 14 days. The minimum dose of stilbestrol producing a maximal increase in uterine weight was 0.08 μg., and the corresponding dose of estradiol was 0.01 μg.

When testosterone was used in female mice, the dose as determined above was arbitrarily doubled, to 40 μg. daily. The dose of estradiol given to male mice was increased to 0.1 μg. daily. The dose of stilbestrol used in the male mice could be increased only to 0.1 μg. daily, because larger doses had a toxic effect.

The main experiment was arranged as follows: there were 70 mice of each sex, divided into 7 groups of 10. One group of each sex was intact, and the remainder were castrated and hypophysectomized; of the latter, one group of each sex was injected with corn oil without any hormone, and one group of each sex with each of the following: testosterone, estradiol, stilbestrol, testosterone plus stilbestrol, and testosterone plus estradiol.

Body weight (Tables 1 and 3)

The males weighed more than the females. All of the mice which were

TABLE 1. AVERAGE ORGAN AND BODY WEIGHTS FROM INTACT FEMALE MICE AND FROM THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED FEMALE MICE. TEN MICE IN EACH GROUP

Numbers are means in mg. ± standard deviation

Organ	Intact	No horm.	Testosterone	Stilbestrol	Estradiol	Stilb.-test.	Estra.-test.
Adrenal	2.76±0.33	0.99±0.10	0.88±0.10	0.90±0.15	0.87±0.14	0.76±0.07	0.79±0.15
Heart	101±7	50±3	52±4	48±2	48±3	49±2	50±4
Kidney	168±17	75±6	103±10	73±5	71±6	97±7	103±10
Liver	1397±127	967±92	1142±81	856±54	865±150	972±102	1081±71
Spleen	80±12	41±9	45±4	41±6	38±5	39±8	36±3
Thymus	35±3.4	26±3.5	14±1.7	29±3.8	27±4.7	10±2.5	19±3.6
Uterine Horn	43±9.6	5.3±0.6	21±2.9	30±4.4	21±3.1	19±3.4	18±2.5
Body (Autopsy)	25.0 Gm.	16.3	17.6	16.0	15.8	16.0	17.1

TABLE 2. STATISTICALLY SIGNIFICANT DIFFERENCES IN ORGAN WEIGHTS BETWEEN THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED FEMALE MICE, LISTED ACCORDING TO HORMONES INJECTED. WHEN THE MEAN ORGAN WEIGHT OF ONE OF THE HORIZONTALLY LISTED GROUPS IS LARGER THAN THAT FROM A VERTICALLY LISTED GROUP, AND P IS LESS THAN 0.01, THAT FACT IS INDICATED BY ++; IF P IS LESS THAN 0.05, BY +. MINUS SIGNS INDICATE THE OPPOSITE RELATIONSHIP

Organ	Animal groups	Animal groups					
		No horm.	Test.	Stilb.	Estra.	Stil.-Test.	Extra.-Test.
Adrenal	No horm.		–			– –	– –
	Testost.	+				– –	
	Stilb.					– –	
Kidney	No horm.		++			++	++
	Stilb.		++			++	
	Estra.		++				++
Liver	No horm.		++	– –			+
	Test.	– –		– –	– –	– –	
	Stilb.	++	++			+	
	Estra.		++				++
Spleen	Test.				– –		– –
Thymus	No horm.		– –			– –	– –
	Test.	++		++	++	– –	++
	Stilb.		– –			– –	
	Estra.		– –				– –
Uterine Horn	No horm.		++	++	++	++	++
	Test.	– –		++			–
	Stilb.	– –	– –			– –	
	Estra.	– –					–

TABLE 3. AVERAGE ORGAN AND BODY WEIGHTS FROM INTACT MALE MICE AND FROM THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED MALE MICE. TEN MICE IN EACH GROUP

Numbers are meanns in mg. ± standard deviation.

Organ	Intact	No horm.	Testosterone	Stilbestrol	Estradiol	Stilb.-test.	Estra.-test.
Adrenal	1.76±0.12	0.82±0.07	0.94±0.15	0.77±0.08	0.77±0.09	0.80±0.09	0.77±0.11
Heart	118±5	53±3	54±2	53±3	56±3	54±4	53±4
Kidney	227±16	89±5	110±7	95±6	99±9	114±10	115±10
Liver	1605±119	917±84	1089±65	956±49	1032±110	964±90	1022±106
Spleen	67±7	34±3	42±3	47±8	48±4	36±5	38±5
Thymus	30±5.0	22±4.9	25±2.1	20±2.2	24±3.4	15±3.3	14±1.6
Seminal vesicle	66±9.9	4.3±0.6	23±3.2	4.1±0.5	4.9±1.0	17±2.4	19±2.2
Body (autopsy)	28.1 Gm.	18.1	18.3	18.7	18.6	17.5	16.3

operated on lost weight, from 13 to 32% between operation and autopsy. Differences between groups of castrated and hypophysectomized mice are not statistically significant. All the intact mice gained weight during the same period.

Vaginal Smears

Castrated-hypophysectomized mice receiving no hormone injections had unvarying, scanty vaginal smears in which there were only a few cells, mostly leukocytes, and little or no mucus. Estradiol or stilbestrol produced an abundance of cornified cells after the third or fourth day.

Testosterone produced increased mucus, "mucified" cells (Fig. 3), and leukocytes after the third day, but no cornified cells were seen.

When both testosterone and estradiol were given, the picture was not distinguishable from that produced by testosterone alone.

TABLE 4. STATISTICALLY SIGNIFICANT DIFFERENCES IN ORGAN WEIGHTS BETWEEN THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED MALE MICE, LISTED ACCORDING TO HORMONES INJECTED. SYMBOLS AS IN TABLE 2

Organ	Animal groups	Animal groups					
		No horm.	Test.	Stilb.	Estra.	Stil.-Test.	Estra.-Test.
Adrenal	No horm.		+				
	Test.	−		−	−	−	−
Kidney	No horm.		++		+	++	++
	Stilb.		++			++	
	Estra.	−	+				++
Liver	No horm.		++		+		+
	Test.	−−		−−		−−	
Spleen	No horm.		++	++	++		
	Test.	−−				−	
	Stilb.	−−				−−	
	Estra.	−−					−−
Thymus	No horm.					−−	−−
	Test.			−−		−−	−
	Stilb.		++			−−	
	Estra.						−−
Seminal Vesicle	No horm.		++			++	++
	Test.	−−		−−	−−	−−	−
	Stilb.		++			++	
	Estra.		++				++

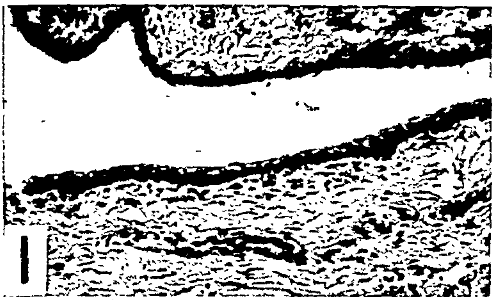
When testosterone was combined with stilbestrol, there was a varying predominance of cornified or mucified cells from day to day in a given animal. Both types of cell were seen, along with mucus and leukocytes in most of the smears (Fig. 4).

EXPLANATION OF FIGURES 1–8

Photomicrographs of tissues from castrated and hypophysectomized female mice. H. and E. stain. ×150.

FIGS. 1–5 Vaginae. 1) No hormone given. 2) Stilbestrol. Epithelium is similar to that in intact mice. 3) Testosterone. 4) Stilbestrol and testosterone. 5) Estradiol and testosterone.

FIGS. 6–8 Uteri. 6) No hormone given. The magnification appears different from that in Figs. 7 and 8, but it is the same. 7) Stilbestrol. 8) Stilbestrol and testosterone. The uteri from mice given testosterone alone, and those given estradiol, or estradiol and testosterone, were similar to Fig. 8.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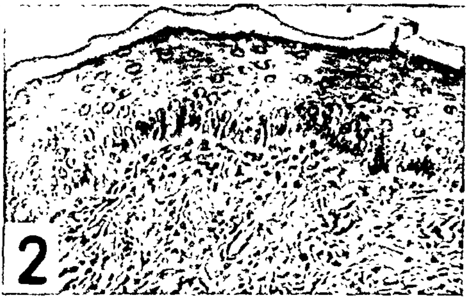
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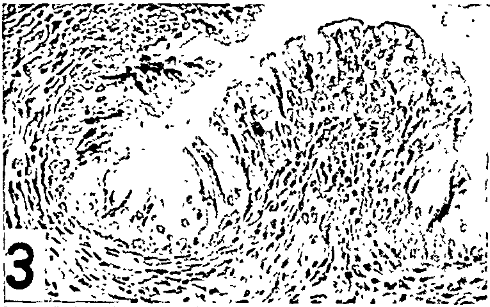
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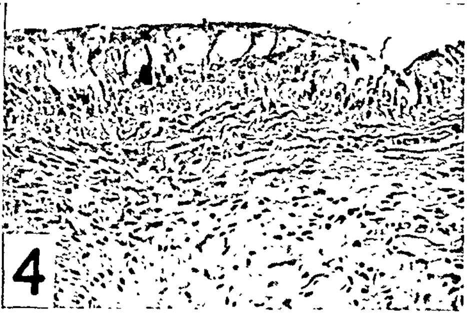
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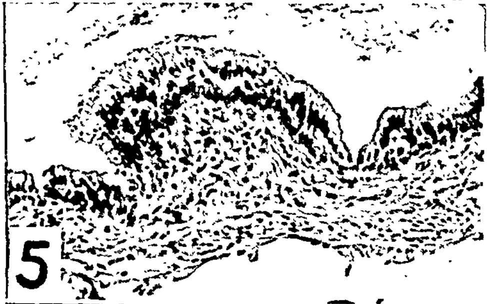
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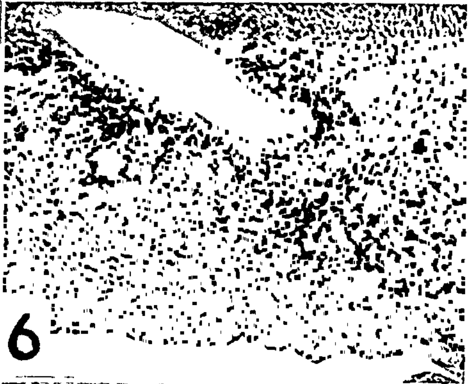
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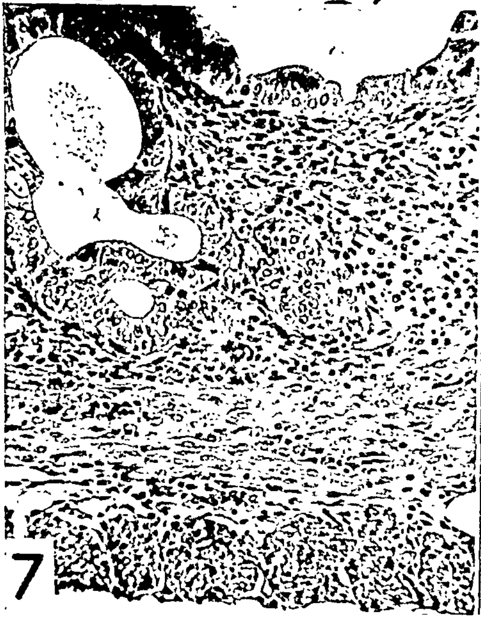
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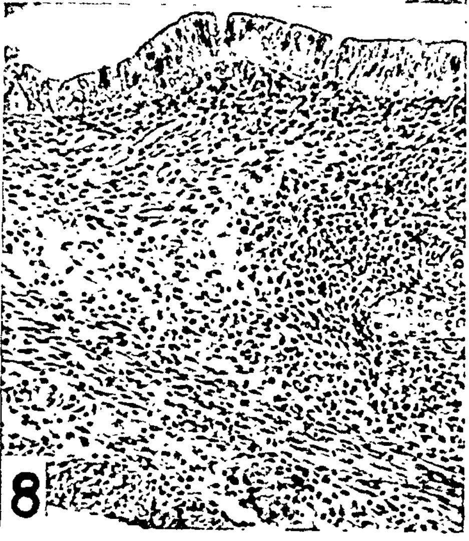
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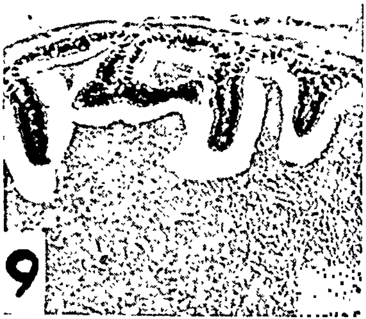
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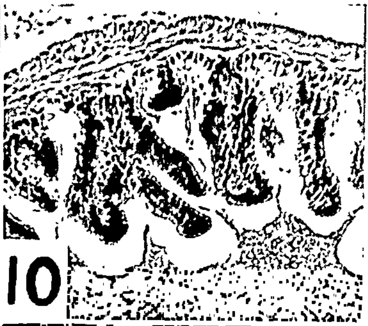
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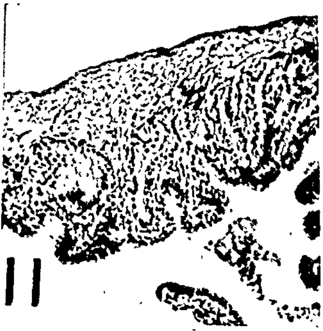
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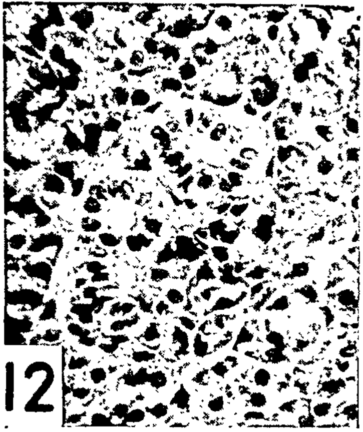
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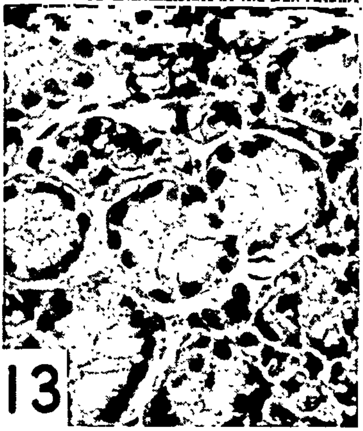
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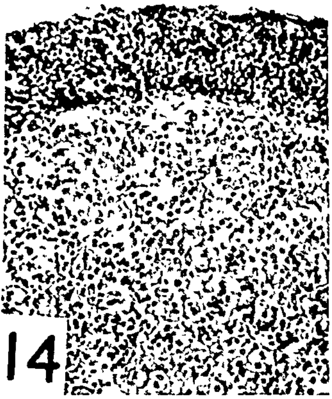
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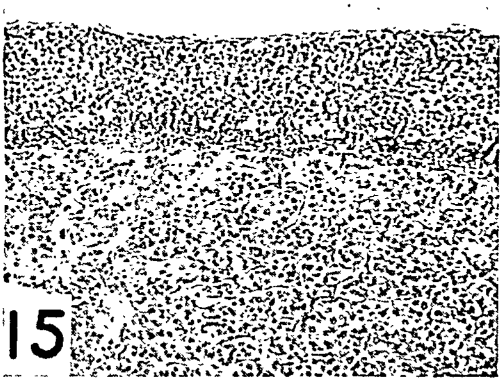
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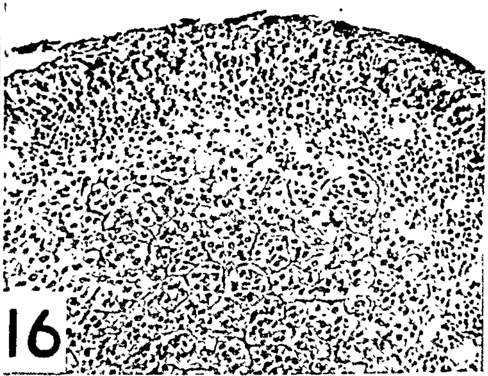
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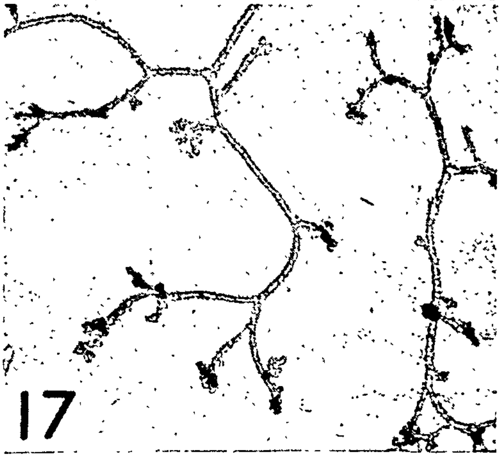
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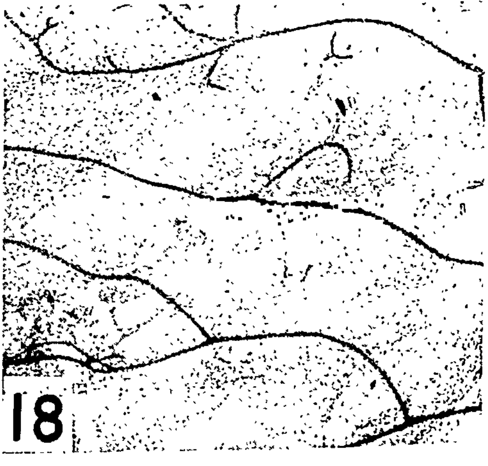
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Changes in organ weights (Tables 1–4)

Uterine horn. The uterus underwent severe atrophy after castration and hypophysectomy. It was restored to nearly $\frac{3}{4}$ normal size by injections of stilbestrol, and to about half normal size by either estradiol or testosterone. A significant inhibition of the effects of either stilbestrol or estradiol was found when testosterone was added; in fact, when both estrogen and androgen were given, uterine weights were less in each case than when either was given alone. The inhibitory effect appeared to be mutual.

Seminal vesicles. These were weighed without loss of secretion, and judging by the histology (Figs. 9–11), the differences in weight are mainly due to differences in the amount of secretion present. Pronounced atrophy as well as decreased secretion occurred after operation. The injection of testosterone produced secretion and a gain in weight to about $\frac{1}{3}$ normal size. This effect was partially inhibited by either estradiol or stilbestrol, neither of which had any effect when given alone.

Adrenals. The sections (Figs. 14–16) show that differences in adrenal weights are mainly due to changes in the cortex. The findings were different in the two sexes: In the female, changes were always in the direction of smaller glands when any hormones were given, and there was more atrophy when both hormones were given than in the other groups. In the male, the only significant change from the untreated controls was a slight increase in weight when testosterone was given alone, which was apparently completely inhibited by simultaneous administration of either stilbestrol or estradiol.

Kidneys. There was a reduction in weight of 55% in the female and 61% in the male after operation. An increase in weight was produced in either

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EXPLANATION OF FIGURES 9–18

Photomicrographs, H. and E. stain. ×150 or 200.

FIGS. 9–11. Seminal vesicles from castrated and hypophysectomized male mice. 9) Given testosterone. 10) Testosterone and estradiol. 11) Estradiol. Seminal vesicles from mice given stilbestrol and from those not given any hormones closely resemble Fig. 11.

FIGS. 12 AND 13. Submaxillary glands from castrated and hypophysectomized female mice. 12) Given stilbestrol. This appearance was found in all groups except intact males and mice given testosterone. 13) Stilbestrol and testosterone. Testosterone alone and testosterone plus estradiol gave the same picture as Fig. 13.

FIGS. 14–16. Adrenal glands from castrated and hypophysectomized female mice. 14) Given testosterone. 15) Stilbestrol. 16) Testosterone and stilbestrol.

FIGS. 17 AND 18. Mammary whole mount preparations ×30. 17) Intact female mouse. 18) Castrated and hypophysectomized female mouse given stilbstrol; those given the other hormones or no hormone had the same appearance.

sex by testosterone, and this effect was not inhibited by estrogen. Stilbestrol alone had no effect, but there was some increase in weight of the male kidney when estradiol was given.

Liver. Liver weight was increased by testosterone, and this effect was inhibited by stilbestrol, but not by estradiol, which by itself produced an increased weight in the male.

Thymus. Weight was decreased in the female by testosterone, and this was unchanged when stilbestrol was added, but the decrease was less if estradiol was added. In the male there was a decreased weight when two hormones were given together, but no effect from any of the three given separately. These changes were not correlated with the differences in adrenal weights.

Spleen. In the male, weights were increased when estrogen or androgen were given alone, but were unaffected when both were given. In females there was little change.

Heart. The heart weight was remarkably constant and unaffected by sex hormones.

Histology

After operation, there was atrophy of nearly all tissues, with a decrease of cytoplasm and closer packing of cells.

Vagina (Figs. 1–5). Following operation there was pronounced atrophy of the entire structure. Epithelium was reduced from about 8 layers of cells to two layers without cornification. When estradiol or stilbestrol was given, the epithelium returned to approximately normal, but the rest of the vaginal wall remained atrophic. Injections of testosterone produced a vaginal mucosa 3 to 4 layers thick, with large pale-staining cells, and with mucus being discharged into the lumen. When estradiol was given with testosterone, the picture was similar to that with testosterone alone. When testosterone and stilbestrol were combined, the pattern varied, with the deeper layers of the mucosa resembling the stratified squamous, and the layer nearest the lumen mucified in most areas.

Uterus (Figs. 6–8). The extreme atrophy occurring in this organ after castration and hypophysectomy is but little more than that occurring after castration alone. There was considerable restoration of all layers after injection of estrogen. Many endometrial glands were formed when stilbestrol was given. This effect was diminished, and muscular development was less evident if testostrerone was added to stilbestrol. Estradiol had less effect on musculature, and glands were smaller than those found after stilbestrol injections. Uteri from animals injected with testosterone, or testosterone and either estrogen were very similar histologically, and are well represented in Figure 8.

Seminal vesicles (Figs. 9–11). The appearance in those mice given no hormone or either estrogen is the same (Fig. 11). Testosterone caused the organ to become distended with secretion, and this effect was decreased when estrogen was added.

Prostate and coagulating glands. The flattened eipthelial cells seen in mice given no hormone after operation were changed to larger, cuboidal cells by testosterone, but were apparently unaffected by estrogen. There was no squamous metaplasia, and estrogen did not modify the effect of testosterone. Squamous metaplasia has been reported in these structures after doses of estrogen considerably larger than used here (Thorborg, 1948).

Submaxillary glands (Figs. 12, 13). Sexual bimorphism in this organ was originally described by Lacassagne (1940). In the present experiment, mice operated on all had the female type of ductule (Fig. 12), except those given testosterone, which all had the male type (Fig. 13). Ductules of the latter were not modified when estrogen was added to testosterone.

Kidneys. The number of Bowman's capsules having cuboidal cells in the parietal layer is increased in the castrate male mouse by injection of testosterone (Crabtree, 1941). In the present experiment it was not possible to find significant differences in the number of such capsules in the various groups, although higher numbers were found in males given testosterone.

Adrenals (Figs. 14–16). All adrenals from animals operated on showed atrophy of the cortex, and this feature was not conspicuously modified by any of the hormones given. The cortex in the male was essentially unchanged in appearance by the various injections. In the female after operation there was a concentration of small dark cells in the inner part of the cortex, about 8 layers thick, representing the atrophied x-zone. This area was not distinguishable in females given testosterone, with or without estrogen, but was present in all others.

The female mice given testosterone with either estrogen showed an uneven basophilic staining of nuclei in both cortex and medulla (Fig. 16). This may represent more rapid atrophy or degeneration, and is correlated with the greater loss of adrenal weight in these groups.

Areas of "brown degeneration," a deposition of ceroid pigment, were found in most of the female adrenals, in the inner part of the cortex. They were most prominent in the group given stilbestrol (Fig. 15).

Other organs. Sections were studied from the following organs without finding significant differences between the various groups of castrated and hypophysectomized animals: liver, pancreas, spleen, sublingual gland, thyroid, parathyroid, trachea, esophagus, thymus, lung, heart. stomach, duodenum, ileum, colon, and bladder.

Mammary whole mounts (Figs. 17, 18)

The mammary glands were removed from the skin of each animal and examined, and complete preparations were made from two animals of each group. There was no development of the mammary ducts in any of the animals which had been castrated and hypophysectomized. Examples of estrogen-androgen antagonistic effects on the mammary gland in intact or castrated animals have been reported (Smelser, 1933; Arbelger and Huseby, 1951), and may have involved pituitary suppression as part of the mechanism; however, the level of pituitary factor necessary for mammary growth might be unaffected by sex hormones, though essential for their action.

DISCUSSION

True hormone antagonism might be defined as a situation where a measured characteristic of a target organ is affected in one direction by one hormone, and is affected only in the opposite direction by addition of the other hormone. Examples of such antagonism in the present study were observed in the amount of seminal vesicle secretion, the weight of the liver, the weight of the thymus in the female, and the extent of the x-zone in the female adrenal cortex.

Other types of effect observed included instances where both sex hormones produced alterations in the same direction, but where one nevertheless inhibited the effect of the other, as in development of the endometrium, uterine weight, and splenic weight in the male, A site where different, but not opposite, effects were exhibited separately by the two sex hormones was the vaginal mucosa. Examples of places where only one hormone showed activity were the ductules of the submaxillary gland, and the weight of the kidney (testosterone versus stilbestrol). Finally, in two instances the two hormones together had a synergistic effect which neither had alone, on thymus weight in the male and adrenal weight and histology in the female.

The idea that all tissues will respond to one sex hormone if they are affected by the opposite one could not be proved or disproved unless an extensive dose range of both hormones were tested; in the range of dosage near physiological, which we have used, this conception did not apply. A theory of universal estrogen-androgen antagonism is not supported by our data.

Pituitary function may affect in some degree the response of various target organs to injected sex steroids, but the essential agreement between the results in this experiment and those reported from non-hypophysectomized animals implies that pituitary hormones are not indispensable to sex steroid action on the target organs studied, except the mammary gland. The latter did not respond to injected steroids after hypophysectomy.

SUMMARY AND CONCLUSIONS

A study has been made in 140 castrated and hypophysectomized C_3H male and female mice, and 20 intact controls, of the effects of injected testosterone propionate, and diethylstilbestrol or α-estradiol, separately and in combination. Injections were begun 31 to 35 days after operation and continued daily for 14 days. Observations were made of vaginal smears, body weight, organ weights, histology, and mammary whole mounts.

The results indicated that antagonistic effects of simultaneously administered estrogen and androgen in the castrated and hypophysectomized mouse are evident, but are not predominant; there are also independent, unlike effects of the two hormones on the same tissue, sites where only one hormone is effective at the given dose level, similar effects by both hormones on the same tissue, and synergistic effects not seen with either hormone alone. The similarity of these findings to those reported in intact or castrated animals suggests that the pituitary does not play a determining role in the mechanism of action of the sex hormones on the target organs studied, except the mammary gland.

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EFFECTS OF CORTICOSTEROIDS ON URINARY FORMALDEHYDOGENIC CORTICOIDS AND 17-KETOSTEROIDS IN NORMAL AND SUBTOTALLY HEPATECTOMIZED RATS[1]

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URINARY outputs of 17-ketosteroids (17-KS) and reducing (R) or formaldehydogenic (F) corticoids (C) are used as measures of adrenal cortical function. However, they are not readily interpretable. Under different conditions, urinary 17-KS and FC can vary in the same sense or independently (Venning, 1950). Among factors contributing to this variability of relationship, the nature of the steroids undergoing metabolism is perhaps the most important. Thus, in human beings (Venning, 1950; Polley and Mason, 1950) and in rats (del Greco, Masson and Corcoran, 1952), only C-17-hydroxylated corticosteroids act as 17-KS precursors; the extent to which injected corticosteroids appear in urine as FC varies widely (Fajans, Louis and Conn, 1951 a). Among other variables are the state of esterification, the route of administration and the level of hepatic function (Fajans, Louis and Conn 1951 b; Engstrom *et al.*, 1951; Bongiovanni and Eisenmenger, 1951). The aim of this study was to test the significance of these factors on absolute and relative outputs of urinary 17-KS and FC.

A method, previously described for the measurement of urinary 17-KS in the rat (del Greco, Masson and Corcoran, 1952), was adapted which depends in principle on giving large doses of a test compound over a short space of time, so that effects of endogenous hormones are obscured. The variables tested in these experiments are (a) the nature of the compound, (b) its esterification, (c) oral as compared with subcutaneous administration, and (d) the effect of subtotal hepatectomy.

PROCEDURE

Female albino rats (150 to 250 gm. body weight) were divided in groups

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[2] Post-doctorate Fellow, Public Health Service.

of at least 4 animals. Each rat was placed separately in a metabolic cage; the animals were maintained on Purina Fox Chow; 1% NaCl was substituted for drinking water in order to increase urine flow.

The pretreatment control period of 4 days enabled the animals to accustom themselves to their new environment; they were then placed on real or simulated treatment for 5 days; this was followed by a recovery period of 4 days; lack of certain steroids sometimes forced us to shorten the treatment period by 1 or 2 days. In experiments involving sub-total hepatectomy, the operation was done on the last pretreatment day.

The corticosteroids tested were cortisone and hydrocortisone, each as alcohol and acetate, and the acetates of corticosterone, dehydrocorticosterone and desoxycorticosterone; free testosterone was injected as a means of validating the 17-KS method. These steroids were given once a day in doses of 10 mg. in water suspension; the subcutaneous dose was contained in a volume of 0.2 cc. and the oral dose was given by gavage in a volume of 1.0 cc.

Urine was collected daily; 17-KS determinations were done on individual Merthiolate-preserved samples by a method of Drekter *et al.* (1952); determinations of pH 1 extractable FC (Corcoran and Page, 1948) were done on chloroform-preserved samples pooled daily from each group. The extraction stages of the procedures were completed on the day of collection; the extracts were then accumulated for simultaneous final colorimetric analyses. The urine samples were grossly free of feces and food.

This manner of handling the samples is important as concerns FC. In a former study (Masson *et al.*, 1952) urines of individual rats were pooled after addition of chloroform and accumulated at 10° C. to a volume of 300 cc., so that several days elapsed before acidification and extraction. Mean FC output of normal rats by this procedure was 0.5 μg. daily. By the present procedure, the mean of 14 observations (urines from 36 individuals) is 11.2 μg. daily, the standard deviation ± 3.7 and the standard error of the mean ± 1. The effect of spleen β-glucuronidase (Viobin) was examined by comparison of FC estimates in 5 pools of fresh rat urine; 2 were extracted at pH 1 without incubation with the enzyme and yielded values of 4 and 3 μg. daily; the 3 samples extracted at pH 1 after 3 days incubation with the enzyme (procedure 1 of Dustan, Mason and Corcoran, 1953) gave negligible yields in 2 cases and value of 1.5 μg. in the other. The difference in yield between the directly extracted and enzyme incubated samples indicates that there had been destruction of FC during the time of incubation; similar destruction which may be bacterial probably accounts for the low values observed in pooled stored urine samples. Since, in contrast with the human (Corcoran *et al.*, 1951; Dustan, Mason and Corcoran, 1953), the yield of FC in rats was not increased by enzymatic hydrolysis, this procedure was not made part of this study.

RESULTS

I. Normal Rats: Oral Administration

Results are summarized in Table 1. Stated control mean value of FC is that described above (11.2 μg.) since determinations in each group proved unnecessarily tedious. Stated mean control levels of 17-KS are those found in each group. Values of each during treatment are noted for each day and also as the mean of the treatment period. The tabulated post-treatment value is derived from analyses of urine collected during the first post-treatment day. To facilitate estimates of the effects of treatment the mean treatment levels (A) are compared with mean control levels (B) and the ratio A/B presented in the table.

By compounds, the following effects were observed:

(a) desoxycorticosterone acetate: increase in FC and no change in mean 17-KS during treatment but an increase thereafter; in terms of the injected steroid, the yield of FC is 0.11%.

(b) cortisone acetate: increases in FC and 17-KS during treatment; FC yield is 0.63%.

(c) hydrocortisone acetate: increases in FC and 17-KS roughly as observed with cortisone acetate; FC yield 0.8%.

(d) cortisone: increases in FC and 17-KS; FC yield 1.07%.

(e) hydrocortisone: increases in FC and 17-KS; FC yield 0.8%. Yields of 17-KS from hydrocortisone and cortisone alcohols are somewhat less than were observed during treatment with their respective acetates.

(f) dehydrocorticosterone and corticosterone acetates do not increase urinary 17-KS; FC was not observed.

(g) testosterone: no change in FC; increase in 17-KS with a net yield of 11% as 17-KS.

The outputs during the first post-treatment days show abrupt remissions of the changes observed during treatment to values approximating or less than those of control periods.

II. Normal Rats: Subcutaneous Administration

Data are summarized in Table 2 in a manner corresponding to that of Table 1. Only 3 compounds were tested.

(a) desoxycorticosterone acetate: in contrast to oral administration, there is no change in FC; changes in 17-KS correspond to those described above.

(b) cortisone acetate: treatment increases FC and 17-KS as during oral administration.

(c) hydrocortisone acetate: increases in FC and 17-KS during treatment; net yield of FC is only 0.19%, which is one-quarter of the yield found after oral dosage; net increase of 17-KS was one-third less than was found after oral dosage.

In contrast with oral dosage, the values found on the first post-treatment day are has high as those found during treatment; decreases to or towards normal values occur during the 3 succeeding days.

III. Subtotally Hepatectomized Rats

Data are summarized in Table 3 in the manner of Table 1.

(1) Effect of the operation: FC increases abruptly and then decreases to subnormal values over the 5 postoperative days; the mean change is an increase. Roughly parallel changes occur in 17-KS, although the mean shows no change.

(2) Effects of steroids: These, with the exception of desoxycorticosterone, were given only orally. In the ratios A/B of FC, net A (treatment mean) is calculated as observed value less the mean postoperative value (25 μg.) in the group. The ratios of 17-KS are not similarly "corrected," because of the rough equality of pre- and postoperative means in the control group.

(a) desoxycorticosterone acetate: orally, FC increases and 17-KS is unchanged, as in normal animals; subcutaneously, it slightly increases FC.

(b) cortisone acetate: the increase in FC is more than twice that found in normal animals, while the increase in 17-KS is the same.

(c) hydrocortisone acetate: again, the increase in FC is roughly twice that found in normal animals; the output of extra 17-KS was not affected by the operation.

(d) cortisone: the increase in FC is roughly 4 times that found in normal animals; 17-KS excretion is not affected.

(e) hydrocortisone: the increase in FC is 3 times that found in normal animals; again, 17-KS output is not altered.

(f) dehydrocorticosterone acetate: no values of FC output during treatment were available from normal animals; however, the augmentation of FC output is similar to that observed after cortisone and hydrocortisone; 17-KS output was slightly increased by this compound in contrast to the observations in normal animals reported above.

(g) corticosterone acetate: only 17-KS was observed; as with dehydrocorticosterone acetate, the output was slightly increased by steroid treatment.

In these experiments, as in those in normal animals given steroids orally, outputs fell to or towards control levels abruptly on cessation of treatment.

DISCUSSION

The mechanism of renal excretion of steroid hormones and their metabolites is obscure. Certain presumptions seem justified. Most circulating steroid hormone is protein-bound and not available for renal excretion by glomerular filtration. The fraction of steroid in free solution, and that conjugated in a relatively water-soluble form, are subject to glomerular filtration. Consequently, the determinant of the urinary output of administered steroids or of steroid metabolites is the concentration of free and combined steroid in plasma water. This function at constant dosage, rate of absorption and protein concentration, will vary inversely with the rate of steroid catabolism. The variables tested in the above experiment can be considered in terms of these considerations.

(a) The Nature of the Compound. Both cortisone and hydrocortisone, either as alcohol or acetate, without regard to the route of administration or the integrity of liver function, increase urinary 17-KS; in normal animals acetates of desoxycorticosterone, dehydorcorticosterone and corticosterone do not. This accords with previous demonstrations, cited above, that C-17-hydroxylated corticosteroids act as 17-KS precursors. In some observations in human beings cortisone has been observed to decrease urinary 17-KS, presumably by suppressing endogenous cortical secretion (Polley and Mason, 1950; Villa, Ballabio and Sala, 1952). However, in our experiments and in clinical observations of increased post-cortisone 17-KS outputs, the doses used have been large.

All corticosteroids tested increased urinary FC. The largest yields followed administration of cortisone and the least that of desoxycorticosterone acetate. Presumably this reflects the more rapid absorption of cortisone and its comparatively large solubility in plasma (Macek, *et al.*, 1952).

The independent variations of FC and 17-KS seen in disease can, from the above observations, be attributed to changes in the composition of adrenal cortical secretion; this secretion is believed normally to contain largely hydrocortisone, a 17-hydroxylated steroid, and corticosterone (compound B) which has no 17-hydroxyl (Reich, Nelson and Zaffaroni, 1950). Changes in the relative proportions of these two constituents would account for the variable relationship of urinary 17-KS and FC; thus, an increase in the corticosterone content would increase FC while 17-KS would be unchanged or decreased. In this connection also, unstable and frequently high outputs of FC are found in many patients with hypertensive disease (Corcoran, Page and Dustan, 1950); the low or absent yields of FC observed during treatment of rats with desoxycorticosterone indicate that these increases in FC output cannot be attributed to increased secretion of desoxycorticosterone-like compounds. Rather, as noted elsewhere (Dustan, Mason and Corcoran, 1953) the increments in pH 1 extractable FC found in hypertension can be ascribed to decreases in the proportion of FC excreted as glucuronide.

(b) The State of Esterification. The comparisons here available are between alcohols and acetates of cortisone and hydrocortisone. Orally, the alcohols yielded somewhat less 17-KS than the acetates in normal rats; outputs of FC were roughly the same with both forms of the steroids. In human beings, however, the orally administered acetates seem more effective in increasing 17-KS than do the alcohols (Fajans, Louis and Conn, 1951 a).

(c) The Route of Administration. As concerns 17-KS, the notable difference between oral and subcutaneous administration is the greater increase elicited by oral hydrocortisone acetate. This difference is even greater in the case of FC; it prevails, in lesser degree, in the increment of FC which follows oral desoxycorticosterone acetate and the lack of effect on FC of this steroid given subcutaneously. Similarly, in human beings, oral hydrocortisone acetate causes greater increments in urinary 17-KS and FC than does intramuscular (Fajans, Louis and Conn, 1951 a). In this regard, Nelson *et al.*, (1952) found that oral dosage with either hydrocortisone or cortisone acetates causes a prompt rise in plasma 17-hydroxysteroid concentration, while intramusuclar dosage has little or no effect. This difference reflects slow absorption from intramuscular deposits dependent on the poor solubility of the steroid esters in body fluids (Macek *et al.*, 1952). This slow absorption has the effect that excretion of FC from subcutaneously deposited corticosteroid continues over several days, whereas the effect of oral corticosteroid on FC disappears on the day after withdrawing treatment.

(d) Effects of Subtotal Hepatectomy. Patients suffering from moderate or advanced hepatic cirrhosis sometimes show increases in urinary RC and FC associated with decreases in 17-KS (Bongiovanni and Eisenmenger, 1951); these abnormalities can be attributed to defective hepatic catabolism of normal or increased amounts of endogenous corticosteroids. On the other hand, normal values of urinary FC were observed by Mason (1951) in a group of jaundiced patients with various forms of liver disease; the same was observed (Butt *et al.*, 1951) in 2 jaundiced patients in whom also cortisone administration elicited roughly "normal" increases of urinary FC and actual decreases of 17-KS. More recently Chapman *et al.*, (1952) found that cortisone treatment did not alter 17-KS excretion in 3 cirrhotics. Thus, experiments in 2 jaundiced and 3 cirrhotic patients suggest that the low outputs of 17-KS in liver disease may be due to decreased hepatic synthesis of 17-KS from precursor steroids; on the other hand, the data of Mason (1951) and of Butt *et al.* (1951) do not agree with the concept that hepatic disease is associated with inadequate degradation of the corticosteroid side-chain. The above experiments in subtotally hepatectomized rats were designed to explore these mechanisms as affected by one type of hepatic insufficiency.

The operation itself caused a transient (3 days) increase in FC with somewhat parallel changes in 17-KS; both presumably reflect the release and conversion of endogenous corticosteroids as a result of trauma.

The effects of exogenous steroids on urinary FC and 17-KS in the presence of experimental hepatic insufficiency can best be observed by comparisons of the ratios A/B as between Tables 1 and 3.

TABLE 1. EFFECT OF ORALLY ADMINISTERED STEROIDS ON 17-KS AND FC IN NORMAL RATS

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
Desoxycorticosterone Acetate	FC	11.2	29	28	15	18	19	22	12	2.0
	17-KS	190	174	157	195	113	230	174	293	.9
Cortisone Acetate	FC	11.2	98	126	46	63	37	74	9	6.6
	17-KS	144	478	441	738	494	384	507	129	3.5
Hydrocortisone Acetate	FC	11.2	86	152	73	73	69	91	5	8.1
	17-KS	73	291	343	189	427	245	299	95	4.0
Cortisone	FC	11.2	66	—	107	108	192	118	13	10.5
	17-KS	112	172	235	417	—	146	242	79	2.2
Hydrocortisone	FC	11.2	120	56	96	92	—	91	7	8.1
	17-KS	114	263	196	192	264	461	275	219	2.4
Dehydrocorticosterone	17-KS	179	167	181	153	216	—	179	166	1.0
Corticosterone Acetate	17-KS	166	170	175	144	—	—	163	179	1.0
Testosterone	FC	11.2	20	13	9	11	15	14	7	1.3
	17-KS	189	949	475	1048	2380	1220	1214	218	6.4

TABLE 2. EFFECTS OF SUBCUTANEOUSLY ADMINISTERED STEROIDS ON 17-KS AND FC IN NORMAL RATS

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
Desoxycorticosterone Acetate	FC	11.2	21	14	14	5	6	12	N.D.*	1.1
	17-KS	199	182	151	256	180	220	197	304	1.0
Cortisone Acetate	FC	11.2	58	66	71	100	67	72	106	6.4
	17-KS	118	440	429	491	333	504	439	453	3.7
Hydrocortisone Acetate	FC	11.2	28	12	47	26	37	30	34	2.7
	17-KS	87	114	285	188	310	290	237	231	2.7

* N.D. = Not detectable.

First, subtotal hepatectomy does not alter new formation of 17-KS from exogenous corticosteroid. In this respect, this experimental insufficiency contrasts with that observed in the 2 patients of Butt *et al.*, (1951) whose 17-KS outputs decreased during treatment with cortisone. Evidently the

TABLE 3. EFFECTS OF ORALLY ADMINISTERED STEROIDS ON 17-KS AND FC IN RATS SUBTOTALLY HEPATECTOMIZED

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
———	FC	11.2	61	31	21	1	9	25	N.D.*	2.2
	17-KS	158	213	154	114	140	215	167	177	1.1
Desoxycorticosterone Acetate	FC	11.2	66	42	49	30	43	46	6	1.87
	FC†	11.2	64	46	36	49	50	49	21	2.14
	17-KS	164	174	132	136	190	299	182	344	1.1
Cortisone Acetate	FC	11.2	98	151	270	294	121	187	5	14.4
	17-KS	175	336	588	560	521	531	507	185	2.9
Hydrocortisone Acetate	FC	11.2	189	296	362	130	64	208	11	16.3
	17-KS	159	408	697	1151	621	555	686	134	4.3
Cortisone	FC	11.2	562	550	500	274	—	471	16	39.8
	17-KS	124	341	455	264	196	257	300	132	2.4
Hydrocortisone	FC	11.2	484	296	292	362	125	312	10	25.6
	17-KS	176	660	599	558	429	422	534	165	3.
Dehydrocorticosterone Acetate	FC	11.2	312	349	300	292	—	313	N.D.*	25.7
	17-KS	134	370	252	182	155	—	239	133	1.8
Corticosterone Acetate	17-KS	172	277	364	316	378	—	334	146	1.9

* N.D. = Not detectable.
† Subcutaneous Administration.

kind or degree of hepatic insufficiency elicited by sub-total hepatectomy is not enough to alter the conversion of corticosteroid to 17-KS; from this it would seem likely that deficient hepatic formation of 17-KS is not an adequate explanation of the low 17-KS outputs seen in chronic liver disease. Rather, the defect may be akin to that which decreases 17-KS output in most debilitating illness.

Secondly, the data show that subtotal hepatectomy increases FC output in response to injections of corticosteroids other than desoxycorticosterone. Since degradation of the steroid side-chain is effected in part by the liver (Schneider and Horstman, 1951; Louchart and Jailer, 1951), the effect of sub-total hepatectomy on FC output can be attributed to defective hepatic degradation of the steroid side-chain. In this respect also, the subtotally hepatectomized rat differs from the 2 patients observed by Butt *et al.* (1951). The data accord with those of Bongiovanni and Eisenmenger (1951), whose patients were observed during illness due to cirrhosis and ascites; the disparate observations of Mason (1950) were made in patients observed because of the presence of jaundice.

SUMMARY AND CONCLUSIONS

Observations of urinary outputs of 17-KS and FC were made in normal and subtotally hepatectomized rats given exogenous corticosteroids in large doses over brief periods.

The data confirm the function of C-17-hydroxylated corticosteroids as 17-KS precursors. The variable excretions of 17-KS and FC in clinical disease can be attributed to variations in the content of C-17-hydroxylated steroid in the secretion of the adrenal cortex.

Among the compounds tested, cortisone alcohol caused the greatest and desoxycorticosterone acetate the least increase in FC. As between the steroid alcohols and acetates, both yielded comparable increments of FC; the alcohols yielded somewhat less17-KS.

Oral, as compared with subcutaneous administration was more effective in increasing urinary FC; this difference was especially marked with hydrocortisone and desoxycorticosterone.

Provisions of exogenous corticosteroids to subtotally hepatectomized rats augment the output of FC, presumably because of impaired hepatic degradation of the steroid side chain; the new formation of 17-KS from exogenous steroid is not impaired. Consequently, while the increase of FC sometimes seen in chronic liver disease can be attributed to defective liver function, the decreases of 17-KS which commonly occur in such conditions may reflect extrahepatic effects of chronic disease.

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THE LAURENTIAN HORMONE CONFERENCE

The Laurential Hormone Conference of the AAAS will hold its 1953 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 6–11. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Mass., for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than *May 15* in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. *Nervous system-hormone interrelationships*
  The central nervous system and stress-induced eosinopenia.
    Dr. R. W. Porter, Univ. of Calif. School of Medicine at Los Angeles
  Studies of brain metabolism and electrical activity in relation to adrenocortical physiology.
    Dr. Hudson Hoagland, The Worcester Foundation for Experimental Biology
  Effects of hormones on cerebral function.
    Dr. D. M. Woodbury, Univ. of Utah School of Medicine
II. *Thyroid Hormone physiology and biochemistry*
  Triiodothyronine in relation to thyroid physiology.
    Drs. J. Gross and R. Pitt-Rivers, National Inst. for Medical Research, London, and State Univ. of New York Medical Center
  Metabolic effects of thyroid hormones *in vitro*.
    Drs. H. A. Lardy and G. Feldott, Univ. of Wisconsin School of Medicine
III. *Comparative endocrinology*
  Endocrine mechanisms in the life of insects.
    Dr. D. Bodenstein, U. S. Army Chemical Center, Maryland
  Hormones produced by neurosecretory cells.
    Drs. Ernst and Berta Scharrer, Univ. of Colorado Medical Center
IV. *Protein Hormones*
  The preparation and chemistry of crystalline insulin.
    Dr. R. G. Romans, Connaught Medical Research Laboratory
  The chemistry of ACTH.
    Drs. E. E. Hays and W. F. White, The Armour Research Laboratories
V. *The role of hormones in blood and blood-forming organs*
  Endocrine factors and radiation-induced lymphoid tumors of mice.
    Dr. Henry S. Kaplan, Stanford Univ. School of Medicine
  Endocrine influences upon the formed elements of blood and blood-forming organs.
    Dr. Albert S. Gordon, Washington Square College, New York Univ.
VI. *Aspects of Clinical Endocrinology*
  Some problems related to ovarian function and to pregnancy.
    Dr. B. Zondek, Rothschild Hadassah Univ. Hospital, Jerusalem
  Clinical studies on electrolyte and fluid metabolism.
    Dr. Rolf Luft, Serafimerlasarettet, Stockholm
  Endocrine-metabolic studies in man.
    Dr. J. W. Conn, Univ. of Michigan School of Medicine

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THYROTROPIN ASSAY BASED ON THE DEPLETION OF IODINE IN CHICK THYROIDS

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THYROTROPIN assays have been based on either the secondary effects due to the increased activity of the stimulated thyroids or on one or more of the specific effects induced on the thyroid gland by the TSH stimulation of the glandular secretory mechanism. The former assays have been predominantly based on the accelerated metamorphosis of the amphibian larvae (Cuyler *et al.*, 1936; D'Angelo and Gordon, 1950), while the latter assays have been based on the increase in thyroid weight (Rowlands and Parkes, 1934; Smelser, 1938; Bergman and Turner, 1939), on the increase in acinar cell height (Starr and Rawson, 1937; Rawson and Salter, 1940; Adams, 1942), on the appearance of a histological response (Jorgensen and Wade, 1941; Ciereszko, 1945), on the increase in the intracellular colloid droplets of the thyroid gland (Dvoskin, 1947) and on the decrease in the thyroid iodine content (Stimmel *et al.*, 1936; Cuyler *et al.*, 1936). Excellent reviews of the existing thyrotropin assay methods have been included in the articles by White (1944), Adams and Beeman (1946) and Albert (1949).

The method presented in this paper is based on the depletion of iodine in the thyroids of chicks. It is dependent on the comparison of the responses obtained from TSH preparations of unknown potencies with the responses elicited by 2–3 levels of a standard TSH preparation run concomitantly.

MATERIALS AND METHODS

White Leghorn cockerels (from several midwest hatcheries) 1–2 days of age, averaging 30–45 gm. in body weight were utilized. These chicks, kept in a brooder at a fairly

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[1] Deceased.

constant temperature, were maintained on a commercial chick starter mash (Purina).

Three different kinds of materials were used as reference TSH standards at the three stages of assay development. Initially, beef anterior pituitary acetone powder, BAP #1, was used. This was then superceded by 2R3 with an assumed TSH potency of 100%. It was a partially purified TSH obtained by alkaline-alcohol fractionation of a beef anterior pituitary acetone powder. Its hormonal contaminants included ACTH (.06 I.U. per mg. by the ascorbic acid depletion method), gonadotropin (approximately 6 Collip units per mg.), growth hormone (approximately 15% of pure beef growth hormone by the 10 day weight gain method in hypophysectomized rats), oxytocin (.03 U.S.P. units per mg.) and pressor (less than 0.03 U.S.P. units per mg.). Finally the U.S.P. Reference Thyrotropin replaced 2R3 as the standard. One U.S.P. thyrotropin unit is that amount of thyrotropin activity contained in 20 mg. of the U.S.P. Reference Substance; in terms of activity, 2.70 mg. of 2R3 is equivalent to one U.S.P. unit.

The samples containing unknown amounts of thyrotropin included 317-51 which was used in the preparation of the U.S.P. Thyrotropin Reference Substance, various TSH fractions, clinical FSH, ACTH, LH and growth fractions as indicated below.

Upon arrival, 100–200 chicks were individually marked with colored dyes, randomly distributed into ten groups of approximately equal size and injected subcutaneously with 0.2 ml. of an appropriate solution. The groups consisted of a control group injected with saline, 2–3 groups treated with 2–3 levels of the standard TSH BAP #1 (2R3 or U.S.P. Thyrotropin Reference Substance) and several groups treated with thyrotropin fractions of unknown potencies (1–2 levels each). The TSH samples were dissolved in saline at approximately pH 7.5 with the aid of 0.1 N NaOH and stored in a deep freeze between injections.

The chicks were injected once daily for three days. On the fourth day, food was withheld very early in the morning. Twenty-four hours after the last injection, the chicks were killed with ether. The thyroids were carefully dissected out, as described by Cope (1938), rapidly weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance and placed in vials containing redistilled 95% ethanol. One to two pairs of these thyroids were stored in each vial so that each group had ten vials for ten iodine determinations. The thyroids were then assayed for the total iodine content according to the method of Walaszek-Piotrowski and Koch (1952), the results being calculated as micrograms of iodine per milligram of wet thyroid tissue.

The iodine responses elicited in the thyroids of the chicks injected with the TSH samples of unknown potencies were compared with the responses of the chicks treated with the reference TSH. Potency ratios and standard errors were calculated by means of the Irwin log dose method (1937). The potencies were reported as percentages of TSH activity of standard 2R3 or as U.S.P. units of TSH per mg. sample.

RESULTS

As the initial source of thyrotropin, beef anterior pituitary acetone powder, BAP #1, was utilized. For injection purposes, it was triturated with saline, brought up to a volume containing 50 mg. of total solids per ml., extracted at a pH of 7.5 for four hours with constant stirring at room temperature and centrifuged. The resultant supernatant containing 30 mg. of total protein per ml. was then used as a reference standard for the assay of TSH. The doses 1.1, 2.2 and 4.5 mg. of BAP #1 gave a graduated decrease of thyroid iodine such that, when the responses (µg. iodine per mg. thyroid) were plotted against the log doses, a straight line resulted. When the dosage of this pituitary standard TSH was extended to include up to 36 mg. of BAP #1, the iodine responses tended to plateau while the thyroid weight responses did not (see Fig. 1). Consequently, 1.1, 2.2 and 4.5 mg. of BAP #1 were used as a standard reference dose, and the thyroid iodine responses were used as the basis for TSH assay calculation.

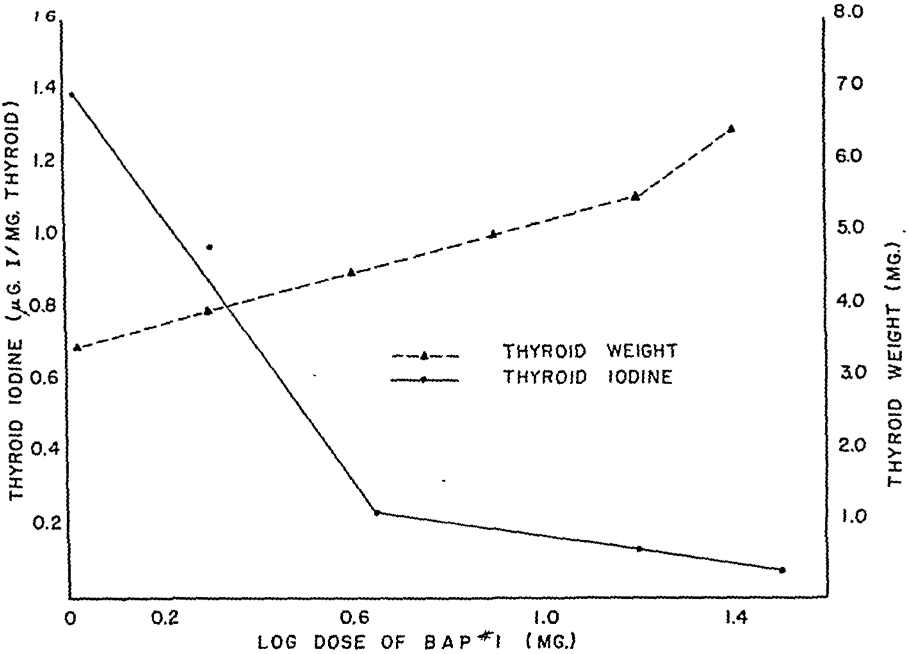

FIG. 1. Depletion of thyroidal iodine and increase in thyroid weight with acetone powder of beef anterior pituitary

As the fractionation of thyrotropin from beef anterior pituitary acetone powder proceeded, a large quantity of purer TSH fraction, 2R3, was obtained. It assayed 2100±600% of BAP #1 and since it was only slightly contaminated with other pituitary hormones, it replaced BAP #1 as the reference standard. A total dose of 80 µg. of this TSH fraction gave a 30–50% decrease in thyroid iodine usually without any thyroid weight response. The total doses finally utilized were 25 µg., 50 µg., and 100 µg. or 40 µg., 80 µg., and 150 µg. A typical assay is shown in Table 1. The samples tested were various thyrotropin preparations with the exception of K4540SR, a clinical FSH sample.

As a final step in this assay development, a U.S.P. Thyrotropin Reference Substance replaced the 2R3 as the reference standard. For purposes

TABLE 1. EXPERIMENTAL TSH ASSAY NO. 165

Control	Standard 2R3			TSH #3	TSH 2+3RW	K45408-R	317-51	U.S.P. TSH	
	0.040 mg.	0.080 mg.	0.160 mg.	0.080 mg.	0.080 mg.	0.530 mg.	0.032 mg.	0.016 u.	0.032 u.
Average Thyroid Weight (mg.)									
3.2_{15*}	3.2_{14*}	3.2_{15*}	3.7_{15*}	3.4_{15*}	3.7_{13*}	3.8_{15*}	3.2_{15*}	3.5_{15*}	3.5_{15*}
Thyroid Iodine Content (μg. I/mg. thyroid)									
1.64	1.43	1.12	0.58	1.28	0.95	0.63	1.01	1.39	0.90
1.33	1.38	0.88	0.44	1.03	0.76	0.52	0.98	1.18	0.81
1.21	1.17	0.81	0.44	0.82	0.66	0.44	0.93	1.16	0.66
1.20	0.92	0.73	0.42	0.71	0.60	0.38	0.57	0.97	0.61
0.97	0.83	0.68	0.41	0.63	0.41	0.35	0.54	0.91	0.60
0.91	0.80	0.57	0.34	0.61	0.40	0.33	0.51	0.69	0.42
0.88	0.74	0.49	0.32	0.57	0.39	0.32	0.48	0.54	0.41
0.81	0.73	0.44	0.31	0.54	0.35	0.22	0.40	0.41	0.28
0.77	0.68	0.39	0.17	0.40	0.32	0.21	0.37		0.27
0.70	0.60	0.23		0.36	0.25	0.15	0.34		
1.04	0.93	0.63	0.38	0.70	0.51	0.36	0.61	0.91	0.55

* Subscripts indicate number of animals per group.

Using Irwin's log dose method for calculation of TSH potencies, the results are:

TSH #3.......................... 88 ± 20% of Standard TSH 2R3
TSH 2+3RW.......................140 ± 33% of Standard TSH 2R3
K45408-R........................ 31 ± 8% of Standard TSH 2R3
317-51..........................280 ± 65% of Standard TSH 2R3
USP.............................300 ± 55% of Standard TSH 2R3

of assay, the doses 0.0125, 0.025, and 0.050 units of U.S.P. TSH have been used (these are approximately equivalent to 40 μg., 80 μg., and 160 μg. of thyrotropin sample 2R3). The 0.025 unit dose decreased the iodine contant of the chick thyroids 30–60% without usually affecting the thyroid weight.

DISCUSSION

Cockerels other than the White Leghorns used were tested for their sensitivity to TSH but were found to be unsuitable. The normal guinea pig and hypophysectomized rat were found to be very much inferior to the two day old White Leghorn cockerel. The guinea pig was found to be especially insensitive to TSH as 2.0 mg. of TSH #2R3 gave no decrease in thyroid iodine and only 35% increase in the mean thyroid weight. On the other hand, a dose of 2.0 mg. TSH #2R3 in the hypophysectomized rat caused a mean 18% decrease in thyroid iodine as compared to hypophysectomized controls and no increase in thyroid weight.

The White Leghorn cockereles have been found, thus far, to be the only breed of chicks very sensitive to thyrotropin. However, there were periods of variation in their sensitivity to TSH. These chicks seemed to be most sensitive in June and August, and least in April, November and December. To avoid variations in responses, a routine of injecting twice daily for three days was studied, but this resulted in responses no different than the one injection per day routine. This could be explained readily because of the prolonged action of TSH on the thyroid (see Table 2), the effect being apparent 72 hours after a single injection of TSH. A one dose assay was then studied. One hundred chicks were injected once subcutaneously with 0.2 ml. solution, autopsied two days later, and the thyroid iodine determinations were performed as described above. The responses were poor. There was a 12% decrease in iodine at the 12 μg. level of 2R3, a 40% decrease at the 50 μg. level and only a 54% decrease at the 3.2 mg. level. Finally the thyroid weight response method was investigated and resulted in poorly graded, variable responses to graded TSH doses (0.4 gm. to 3.2 mg.). Consequently, the TSH preparations, in this laboratory, have been assayed using the iodine depletion of the thyroids of two day old White Leghorn cockerels as described above.

TABLE 2. THYROID IODINE OF CHICKS INJECTED ONCE WITH 3 MG. OF 2R3 AND AUTOPSIED AT VARIOUS INTERVALS

Hours after injection	Number of chicks	Average thyroid weight, mg.	Average* thyroid iodine, γ/mg.
0	10	3.6	2.26
0.5	10	3.4	2.39
1	10	3.8	2.25
2	10	3.5	1.96
3	10	4.7	1.71
4	10	4.3	1.65
7	10	4.3	1.59
24	10	3.4	1.46
32	10	3.3	1.41
48	10	3.4	1.27
72	10	4.2	1.31
72 (Control)	10	3.9	2.20

* One determination on the pool of all the thyroids of one group.

To note whether the thyrotropin potencies as obtained by this method were duplicable, two samples of TSH #317-51 and TSH #3 were subjected to several TSH assays over a period of several months using the chicks from one source (Berry's Hatchery, Illinois). These two TSH fractions were of different degrees of purity and activity; TSH #3 was a by-product of TSH fractionation while #317-51 was used in the preparation of the U.S.P. Thyrotropin Reference Substance. The results are shown in Table 3. The weighted mean standard errors obtained were 14.5% for TSH #3 and 7% for 317-51.

To note if the TSH contamination of other pituitary products could be detected by this method, a series of crude and potent hormonal fractions (other than TSH) were assayed. The results obtained (see Table 4) indicated that this assay method was very sensitive and could detect TSH contamination in anterior pituitary hormonal fractions at levels as low as 0.3% of 2R3.

TABLE 3. THYROTROPIN ASSAY RESULTS ON TSH #3 AND 317–51

Sample	Assay No.	Reference standard	Levels of sample per assay	TSH potency	
				% of 2R3	U.S.P. u./mg.*
TSH #3	160	2R3	1	170 ± 78	
	161	2R3	1	70 ± 34	
	162	2R3	1	93 ± 43	
	165	2R3	1	88 ± 20	
	166	2R3	1	103 ± 26	
Weighted Mean				89 ± 13	
317–51	149	2R3	2	270 ± 78	1.0 ± 0.3
	150	2R3	2	350 ± 140	1.3 ± 0.5
	153	2R3	1	180 ± 30	0.7 ± 0.1
	154	2R3	3	220 ± 28	0.8 ± 0.1
	165	2R3	1	280 ± 65	1.2 ± 0.3
	166	2R3	2	250 ± 43	1.0 ± 0.2
	167	2R3	1	340 ± 65	1.4 ± 0.3
	168	USP	1		0.6 ± 0.2
	171	USP	2		1.3 ± 0.4
	172	USP	2		0.9 ± 0.3
	173	USP	2		1.6 ± 0.2
	174	USP	2		1.4 ± 0.2
Weighted Mean				242 ± 17	1.1 ± 0.06

* 2R3 contains 0.37 U.S.P. units per milligram.

TABLE 4. TSH CONTENT OF SOME PITUITARY SUBSTANCES

Sample	% of standard TSH 2R3
Fresh beef anterior pituitary	0.8 ± 0.1
Beef anterior pituitary, acetone powder	5.0 ± 0.2
Sheep pituitary, acetone powder	1.7 ± 1.0
Fresh hog pituitary	1.0 ± 0.4
Hypothalamic extract	0
Prolactin, 2R-CR[1]	0
LH, FW-234[2]	9.7 ± 3.3
FSH, PFC-7C[3]	35.0 ± 11
Growth hormone 22KR2[4]	3.4 ± 3.1
ACTH 58-9(50)[5]	0.3 ± 0.1

[1] Contained 29 ± 8 International units of prolactin per mg.
[2] Assayed 113 ± 19 Collip units per mg.
[3] Assayed approximately 250 Collip units per mg.
[4] Armour's growth hormone standard having a potency of 100%.
[5] Assayed 1.8 International units ACTH per mg.

SUMMARY

A thyrotropin assay method based on the depletion of thyroid iodine has been developed which is dependent on a comparison (by means of the log dose method of Irwin) of responses obtained from unknown TSH preparations run at one or two levels to the responses obtained from two to three levels of a standard thyrotropin preparation. The two level assays gave TSH potencies with a standard error of approximately 25%.

Acknowledgment

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CORRELATION OF PREPARATIVE HISTORY AND METHOD OF ASSAY OF CORTICOTROPIN WITH CLINICAL POTENCY

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INTRODUCTION

IT IS well known that different ACTH assay methods give different results, but the reasons for this have not been fully elucidated.

Li (1952) has pointed out the discrepancies in evaluation of ACTH activity by different assay methods, which include the adrenal ascorbic acid depletion test of Sayers *et al.* (1948), the adrenal weight maintenance, and the hypertrophy and repair tests. Reinhardt *et al.* (1951) have emphasized the importance of applying several assay methods in the study of various ACTH preparations and the importance of absorption rates in assay methods. Discrepancies in potency estimates by the various assay means have also been noted by Young *et al.* (1951), Dixon *et al.* (1951) and Moyer *et al.* (1952). From the evidence presented there has frequently risen the postulate of two factors, one of which effects adrenal weight, and the second depletion of adrenal ascorbic acid.

Bruce, Parkes and Perry (1952) have studied the thymus involution response of nestling rats to ACTH and elaborated an assay method based upon this criterion of activity. Their method utilized litter-mate nestling rats, three daily injections of ACTH suspended in an oil-beeswax medium, and autopsy to obtain thymus weight twenty-four hours after the last injection.

In comparing the results of assay methods involving thymus or adrenal weight with the results obtained by the Sayers technique it must be borne in mind that the Sayers method utilizes intravenous injection of test doses while most other methods utilize subcutaneous or intramuscular injection.

There has been little clarification of the relative importance of routes of administration when different results have been obtained by different methods of assay. In addition, the variable results obtained by the several methods of assay have not been correlated with clinical activity. An abstract of the preliminary phase of these studies was reported by Fisher and Thompson (1952).

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MATERIALS AND METHODS

An experimental, purified ACTH in 16% gelatin standard was prepared for subcutaneous assay purposes. The term "purified" denotes preparations adsorbed and eluted from cellulose by methods originating in our laboratory and by the method described by Astwood *et al.* (1951). This standard was prepared by mixing equal unitage from each of three lots of purified ACTH in gelatin. These lots had been clinically assayed by several investigators and each was found to be two to four times as potent in terms of intravenously assayed International Units as other ACTH preparations in gelatin when administered subcutaneously or intramuscularly. On the basis of the assays on each lot by the intravenous method, and on the basis of intravenous assay of the finished mixture, the experimental standard contained 40 ± 4.0 I.U./cc. The standard has been stored frozen in small aliquots with no indication of potency change over a period of 12 months.

All test solutions contained the required dose of ACTH in 16% partially hydrolyzed U.S.P. gelatin (gel point about 23° C.) containing 0.5% phenol. All test samples in a given experiment were diluted with the same lot of gelatin.

The ACTH preparations used for all experiments reported (except when specified) in this study were derived from hog pituitary glands and were assayed in terms of I.U. to a standard error of 10% or less by the method of Munson *et al.* (1948) using hypophysectomized rats. All doses in subcutaneous experiments were given in terms of I.U.

The ACTH preparations referred to as crude have activities of less than 2 I.U./mg. as contrasted to the purified preparations, isolated by several different adsorption procedures, with activities between 20–60 I.U./mg. Preparations referred to as Type I forms are those which have been subjected to limited hydrolytic conditions during the process of isolation and the Type II and III forms are those subjected to more rigorous hydrolytic treatment, pepsin and pepsin plus acid, respectively. Types II and III are differentiated from Type IV in that the latter form has not been exposed to hydrolytic conditions.

The test animal in all cases was the Sprague-Dawley strain of rat. Intact and hypophysectomized animals of various ages were used as specified within each experimental design.

When adrenal ascorbic acid depletion was utilized as the end response following subcutaneous injection, individual groups of animals were sacrificed at suitable intervals after injection to obtain either a time response pattern or response at a specified time after administration of the hormone.

Preliminary experiments indicated that ACTH preparations prepared by different procedures were different in relative potency when assayed by the intravenous technique as compared to the subcutaneous technique employing either an aqueous or gelatin medium. Utilizing a gelatin medium, a greater response was obtained with relatively slight effect on the time pattern. A gelatin medium was employed throughout the following experiments because of the magnification of effect which could be obtained and because standardization of ACTH in gelatin was a primary problem.

All adrenal weight and thymus weight experiments reported in the present study involved the injection of 0.5 cc. test solution twice a day (9:00 A.M. and 4:00 P.M.) for three days, and autopsy the forenoon of the fourth day.

RESULTS

Figure 1 illustrates the results of a comparison of the adrenal ascorbic acid depleting activity of crude Type IV and crude Type I ACTH in saline, and in gelatin, after subcutaneous injection of 1.0 I.U./rat. Each point on the graph represents the average response of four rats. It may be seen that a gelatin medium produces a marked increase on the response to both types of preparations. However, the effect on the response to Type I ACTH appears considerably greater.

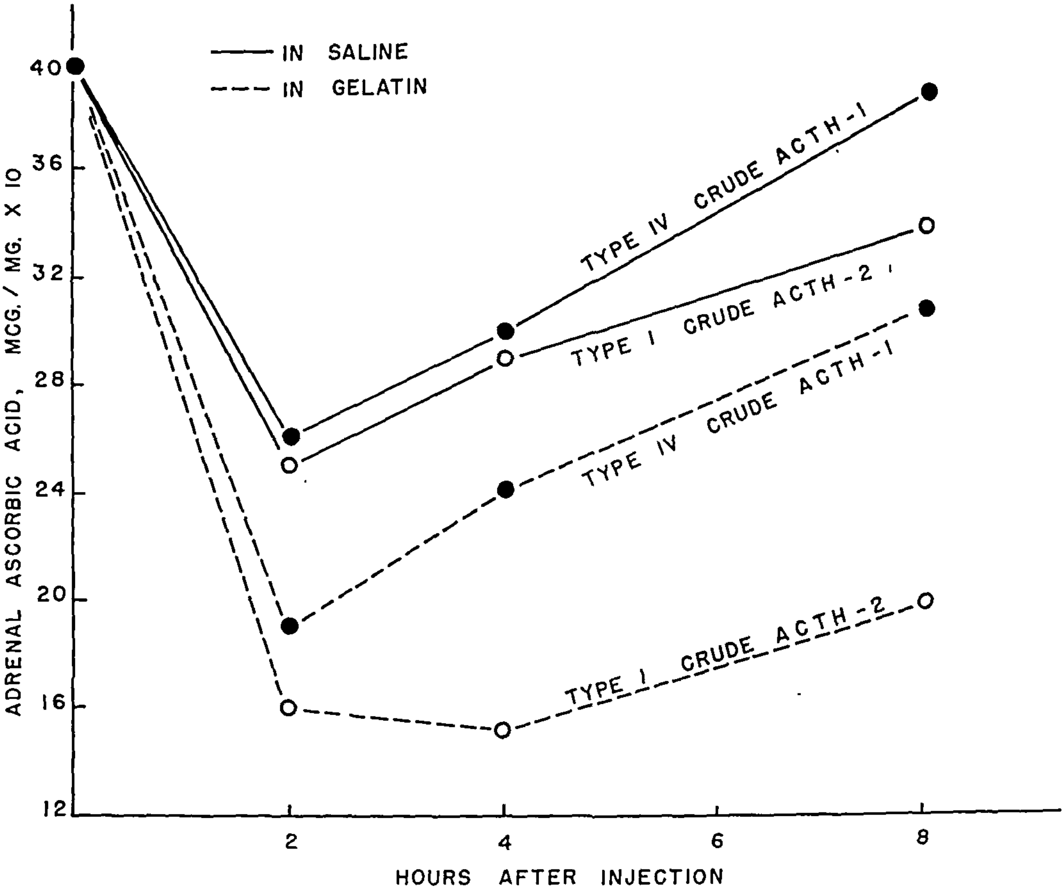

FIG. 1. Adrenal ascorbic acid response versus time after subcutaneous injection of two ACTH preparations in aqueous saline or gelatin vehicle. Each rat received 1.0 I.U. in 0.5 cc. Each point is the mean response value from four hypophysectomized rats.

In order to determine the relative importance of "purity" as compared with "preparative history" in obtaining higher subcutaneous potency in a gelatin medium, the method was converted to a quantitative design and several suitable ACTH samples were assayed in comparison with each other. Figure 2 illustrates graphically the results of such an experiment. Rats were sacrificed at 2 and 4 hours after injection for determination of adrenal ascorbic acid. Since there was no significant difference between the 2 and 4-hour response, these values were combined to produce the results illustrated in Figure 2. Each point then represents the average response of 8 rats.

In Figure 2, the ACTH sample identified as "Purified ACTH-247 (Type I)" assayed 30 I.U./mg., and the sample identified as "Crude ACTH (Type IV)" assayed 1.7 I.U./mg. When these samples in 16% gelatin medium were assayed clinically by intramuscular injection (Wolfson, 1952) the "Purified ACTH-247 (Type I)" was found to be two to four times as potent as the "Crude ACTH (Type IV)." It appears that there is good correlation between this assay method and clinical effectiveness. The sample identified as "Crude (Type I) ACTH-247" is the relatively crude extract (1.0 I.U./mg.) from which "Purified ACTH-247 (Type I)" was derived by selective adsorption. The sample "Purified

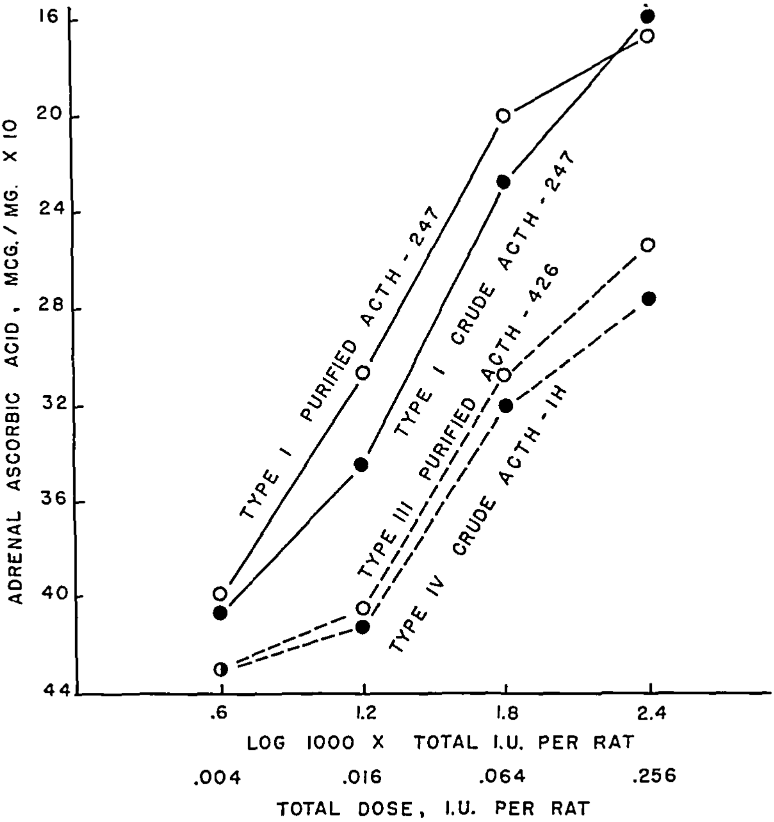

FIG. 2. Assay of several ACTH preparations subcutaneously administered and measuring adrenal ascorbic acid depletion in hypophysectomized rats—eight rats per point—three hour response.

ACTH-426 (Type III)" isolated by chromatopile partition (White *et al.*, 1951) assayed 50 I.U./mg.

In view of the apparent correlation between clinical assays (subcutaneous or intramuscular administration) and the potency value obtained utilizing the adrenal ascorbic acid depletion method (subcutaneous administration), it was anticipated that other subcutaneous methods utilizing normal rats and/or other criteria of activity such as adrenal weight or thymus weight might also correlate with clinical activity.

An experiment on 23 day old normal Sprague-Dawley rats, selected at

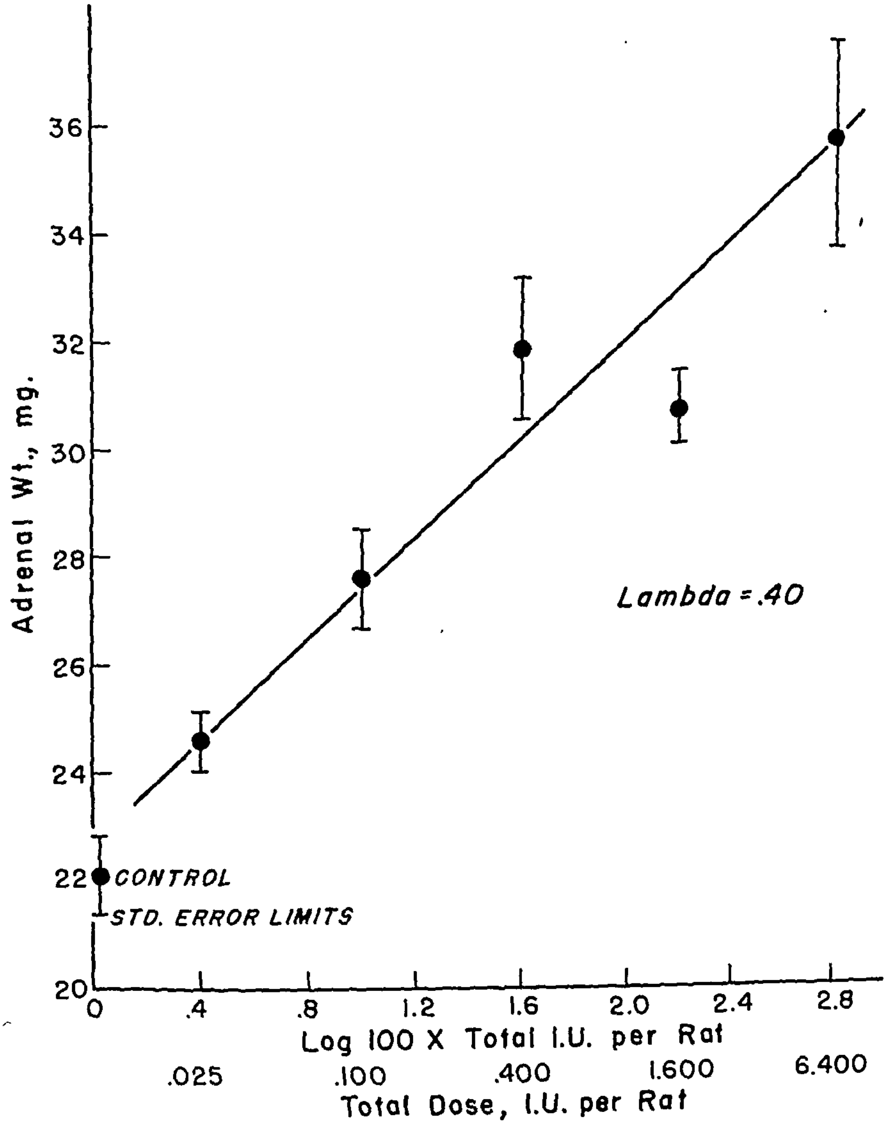

FIG. 3. Adrenal weight response of 23 day old, normal female rats to graded doses of purified ACTH in gelatin. 0.5 cc. subcutaneously twice a day for three days, autopsy fourth day, seven rats per point.

random, indicated that a useful adrenal weight response could be obtained on these animals but that the thymus involution response was more quantitative. Information from both responses could be obtained from the same rats (see also Hayashida and Li, 1952). One similar experiment on 30 day old rats indicated an adequate range of response using either adrenal weight or thymus weight but precision of the thymus response was poor in this experiment as compared to the 23 day old rats. Average control thymus weight was 323 mg. in this experiment and was reduced to 140 mg. with the highest dose of ACTH used (0.40 I.U. purified ACTH (Type I)—total dose in 3 days). Adrenal weight precision was comparable to results from 23 day old rats (lambda = .45).

Figure 3 illustrates the log-dose adrenal weight response relationship of purified ACTH (Type I) in gelatin administered as previously indicated to 23 day old normal female rats. The lambda value of 0.40 indicated relatively poor precision but is sufficiently valid to obtain information with

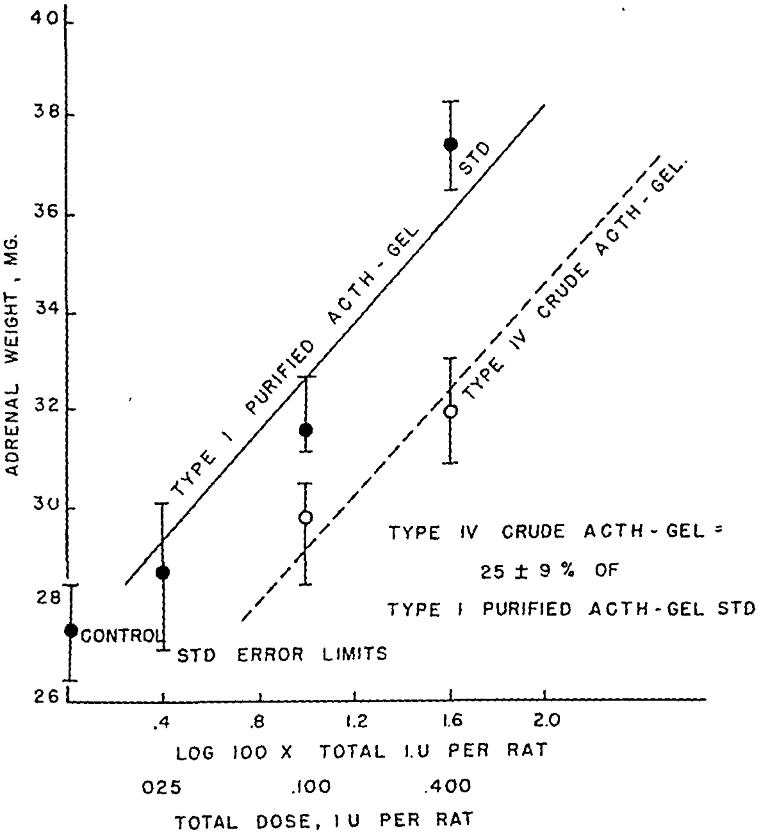

Fig. 4. Adrenal weight assay of ACTH. Thirty day old, normal, female rats—eight rats per point.

respect to correlation between adrenal weight assays and clinical or other assays.

Figure 4 illustrates the application of the adrenal weight response of normal 30 day old rats to the assay of crude ACTH (Type IV) in gelatin as compared to purified ACTH (Type I) in gelatin. The result—Type IV crude ACTH = 25 ± 9% of purified ACTH—is sufficiently accurate to indicate good correlation with clinical assay.

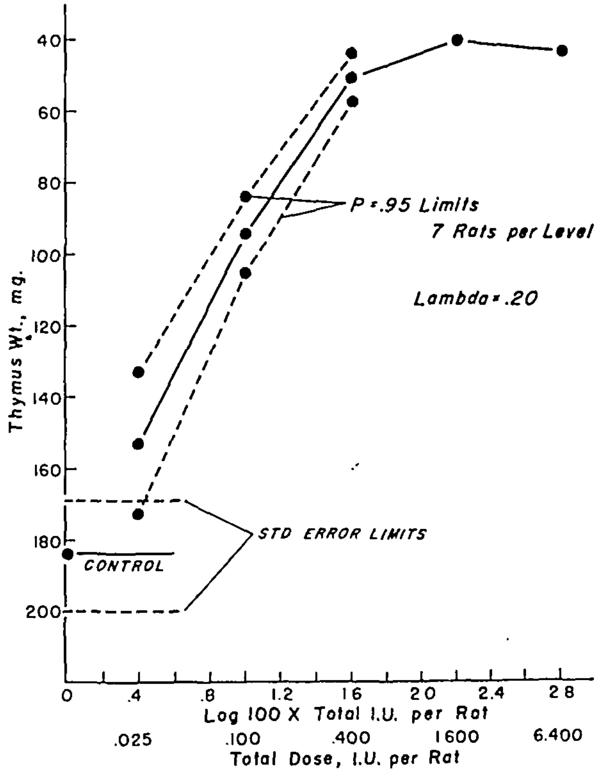

FIG. 5. Thymus weight response of 23 day old, normal, female rats to graded doses of purified ACTH in gelatin. This experiment also provided adrenal weight data shown in Figure 3.

Figure 5 illustrates the log-dose thymus weight relationship of purified ACTH (Type I) in gelatin administered to 23 day old female Sprague-Dawley rats selected at random. The thymus weights were obtained from the same rats that yielded the adrenal weight data illustrated in Figure 3. Thus it can be seen that in the dosage range of 0.10 to 0.40 I.U. of purified ACTH (Type I), quantitative data can be obtained simultaneously from both adrenal and thymus weight responses. In view of the correlation of standard deviation with level of response, conversion of the responses to log-response would seem indicated for assay purposes.

Figure 6 illustrates the assay curve obtained by using the logarithms of the thymus weight response values.

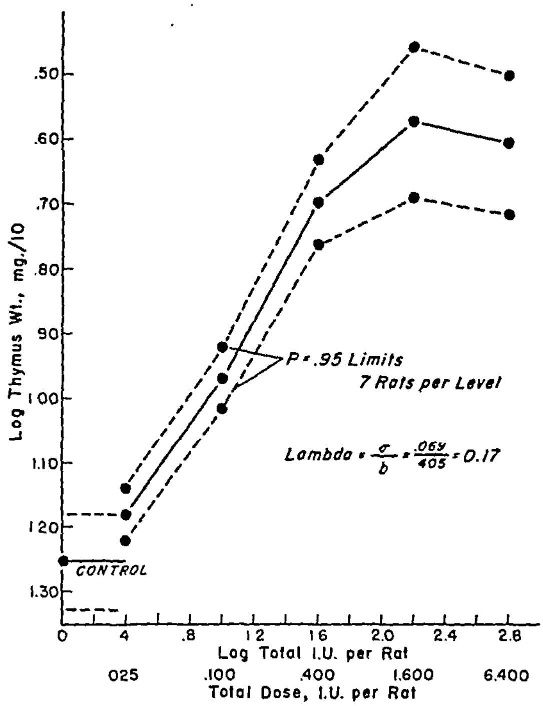

FIG. 6. Log thymus weight plotted against log dose of ACTH using data from experiment illustrated in Figure 5.

Figure 7 illustrates the application of the thymus involution assay procedure for the potency comparisons of several ACTH preparations. Again the comparison of crude ACTH (Type IV) in gelatin with the purified ACTH (Type I) in gelatin shows good correlation with clinical findings and with other subcutaneous assay methods.

In order to study further the application of several assay methods to the problem of ACTH standardization, additional assays were conducted on several samples of purified ACTH (Type I) in gelatin and crude ACTH (Type IV) in gelatin. All samples were first assayed by the provisional U.S.P. adrenal ascorbic acid depletion method on hypophysectomized rats to a standard error of ±10% (150 to 200 rats per sample). For subcutaneous assays, doses were always given in terms of I.U. or U.S.P. Units, so that all preparations can be considered to be 100% of each other, by the intravenous assay technique, within the limits of error indicated above.

Table 1 gives the results of the assay of several lots of purified ACTH (Type I) in gelatin and several lots of crude ACTH (Type IV) in gelatin when utilizing adrenal ascorbic acid depletion following subcutaneous administration in the hypophysectomized rat. It may be noted that each female rats. The average lambda value in these experiments was 0.23 (0.20 to 0.31) so the individual assays are subject to a standard error of about ±16% and the means to a standard error of about ±8%. The purified ACTH (Type I) versus crude ACTH (Type IV) ratio of 3.2, therefore, exhibits close correlation with clinical results and with the other subcutaneous assay methods.

Table 3 gives the results of several assays on crude ACTH (Type IV) in gelatin assayed against the purified ACTH experimental standard by utilizing adrenal weight increase obtained in normal rats. Lambda values for these assays were 0.4 to 0.5 so the individual assays are subject to standard errors of about ±32% but the means are accurate to a standard error of about ±16%. By this method the relative subcutaneous versus intravenous potency of purified ACTH (Type I) in gelatin is about three times that of crude ACTH (Type IV) in gelatin.

TABLE 3. ASSAY OF SEVERAL ACTH PREPARATIONS, SUBCUTANEOUSLY ADMINISTERED, AND MEASURING ADRENAL WEIGHT INCREASE IN NORMAL RATS.

Type IV crude ACTH in gelatin lot	Number or rats		Potency ratio versus type I purified ACTH in gelatin experimental standard.
1	42		.28
1	48		.25
2	42		.37
U.S.P. standard in gelatin			
	42		.35
		Mean	.31

1.0 I.U. purified Type I ACTH in gelatin = 1.0/.31 = 3.2 I.U. crude Type IV ACTH in gelatin.

DISCUSSION

The results indicate that if crude ACTH (Type IV) in gelatin were assayed by a subcutaneous method against the present U.S.P. standard, about the same value would be obtained as at present with the intravenous assay procedure. If the purified ACTH (Type I) in gelatin were assayed against the U.S.P. standard by the same method, a relative potency of about three times that of the intravenous assay technique would be obtained. This corresponds to the clinical assay of these two types of ACTH preparations. The inference is that, if preparations were assayed against the present U.S.P. standard by a subcutaneous method, the resulting products would be uniform in clinical activity when labeled in terms of U.S.P. units and used subcutaneously or intramuscularly. The clinical doses in terms of U.S.P. units would then be the same for either preparation. If such preparations were now labeled in terms of U.S.P. units as determined by the provisional U.S.P. intravenous assay procedure, one U.S.P. unit of one preparation would be about three times as potent clinically as one U.S.P. unit of another preparation, even though each preparation utilizes a gelatin vehicle. ACTH preparations in gelatin equivalent by the provisional U.S.P. assay are, therefore, not necessarily equivalent clinically following subcutaneous administration.

Some evidence exists (Fisher, 1953) that effective ranges of 0.5 to 3 times the U.S.P. unit may be encountered when comparing different type preparations. An experiment was designed to determine whether differences in dose response curve slopes or differences in time response pattern would be detectable after intravenous administration of Type I and Type IV ACTH preparations. The method used was the same as the Munson *et al.* (1948) assay procedure with the exception that rats were sacrificed at several time intervals. This experiment failed to show a difference between the two preparations comparable to that in the subcutaneous experiments. The time-response patterns appeared identical within limits of error. If a time difference in dose response curve slope exists, the purified Type I preparation shows the flatter slope. This latter feature is the opposite of findings after subcutaneous administration of the preparations. Comparison of the results of this experiment with the result of subcutaneous assays indicated that the major difference in relative potency of the two ACTH preparations appeared only after subcutaneous or intramuscular administration. The difference, therefore, must be due to a difference in rate of absorption or a difference in the rate of extravascular destruction.

The adrenal ascorbic acid depletion method, when ACTH is subcutaneously administered, discriminates quite sharply between preparations. The discrimination appears chiefly in the form of both deviation from parallelism of the log-dose response curve (Fig..2), and differences in time-response pattern (Fig. 1).

All characteristics of the thymus involution experiment in Figure 5 indicate that this could be the basis for a simplified and more economical assay for ACTH than any previously available. Precision appears excellent in spite of the absence of litter-mate distribution. The method also retains the feature of discrimination between preparations in respect to their relative subcutaneous potencies. While only results obtained from the Sprague-Dawley strain of rats have been reported, other strains, Wistar for example, have proven satisfactory.

Response range is adequate. Even for randomly selected rats, precision (lambda = 0.23) appears as good as, or better than, other methods. The use of litter-mates may increase precision.

Since there appears to be a difference in the relative potency of different

TABLE 1. AN ASSESSMENT BY 3 OBSERVERS OF THE DEGREE OF PROPTOSIS OF THE EYEBALLS IN A GROUP OF CORTISONE TREATED RATS AND THEIR CONTROLS

0 = No proptosis. x = Mild. xx = Moderate. xxx = Marked. xxxx = Gross.

Animal No.	Degree of proptosis present			Cortisone
	Observer			
	K.A.	S.M.G.	R.L.	
6	0	0	0	No
38	0	0	0	No
14	xx	xx	xx	Yes
7	0	x	0	No
13	xx	x	x	Yes
85	0	x	x	No
46	0	0	0	Yes
30	xxxx	xxxx	xxxx	Yes
47	x	x	0	Yes
55	0	0	0	No
23	0	0	0	No
29	xx	xxx	xxx	Yes
111	xxx	xxx	xx	Yes
112	x	0	0	Yes
77	0	0	0	No
51	0	0	0	No
104	xx	xx	xx	Yes
103	0	0	0	Yes
63	x	xx	x	Yes
22	0	0	0	No
39	xx	x	x	Yes
62	xx	xxx	xx	Yes
69	0	0	0	No

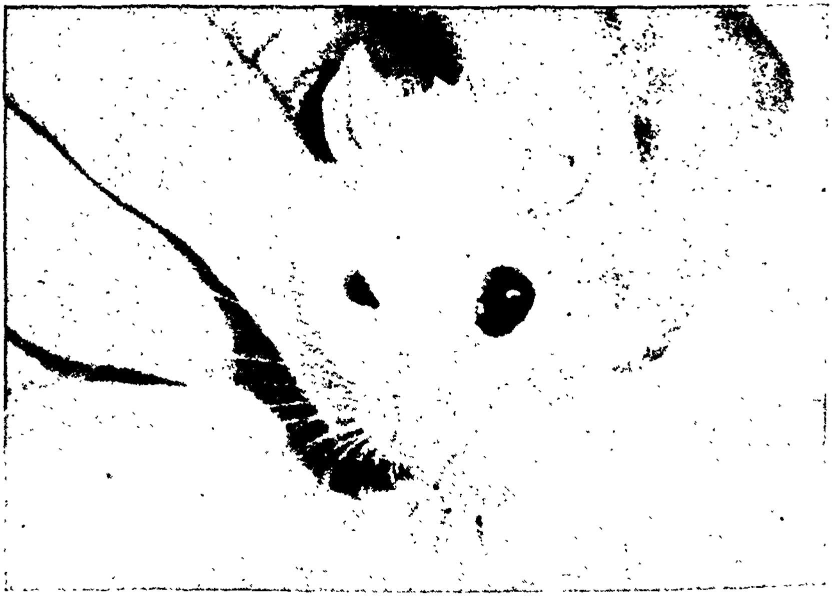

FIG. 2. Gross unilateral ocular protrusion of left eye with only mild proptosis of right eye in a male rat treated with cortisone.

metrical, and no other eye signs, such as the "bloody ring" described by Yudkin, Farquhar and Wakeman (1934) were observed. Proptosis was still present in many animals at the time of autopsy; in at least 4 animals, however, a previously established protrusion had disappeared.

Of particular interest was the course of exophthalmos in one animal, No. 30, to which reference has already been made. Proptosis was diagnosed in this animal by the 3rd day after the start of the cortisone injections. On the 11th day, nothing unusual was noticed, but on the 13th day, the left eye had literally left its socket (Fig. 2). Only moderate proptosis was present on the right side. The left eye as a whole appeared enlarged and close inspection revealed a certain degree of keratoconus. Of great interest was the occurrence here of the unilateral "bloody ring" noted by Yudkin *et al.* (1934) in some of their proptotic animals, a change which is presumably identical with the sanguineous discharge described by Thompson (1933). Pressure on the eyeball did not seem to cause any discomfort. The following day keratoconus was definite; the cornea had become completely dry and opaque; and the proptosis was unchanged. The picture was suggestive of a retroorbital tumour, and it was decided to kill the animal to avoid the possibility of a secondary infection preventing a correct diagnosis. The eye was punctured under light ether anaesthesia and clear fluid escaped under pressure, presumably from the anterior chamber. This undoubtedly was the cause of the keratoconus and the apparent enlargement of the eye, but it did not account for the proptosis, which still persisted. Enucleation showed that the eyeball was not appreciably enlarged. The retroorbital contents appeared hyperaemic, but no tumour or pus could be found. This was confirmed by histological examination (Fig. 3).

This peculiar picture was not observed in any other rat in this or any other series of similar experiments. It is possible that it is similar to the rare unilateral exophthalmos of endocrine origin in man.

DISCUSSION

Yudkin *et al.* (1934) observed proptosis in young male albino rats that were being given diets low in salts. Of 34 rats 11 developed marked and 11 slight and occasionally transient ocular protrusion. A "bloody ring around one eye" was noted in 4 animals. No explanation for this unilateral occurrence is given. On histological examination no evidence of increased ocular tension could be found, nor were there any changes present in either the eye muscles or the retrobulbar tissues. The thyroid is said to have shown "striking changes." Loss of hair was recorded in some animals, but no abnormality was reported in the pituitary or the adrenals, the weights of which, however, are not given.

Somewhat similar findings have been reported by Thompson (1933), who kept rats on diets with varying calcium and iodine contents. Ocular disturbances occurred in the form of proptosis and decreased frequency of blinking. Occasionally a sanguineous exudate developed around the lids of one or both eyes. Proptosis became noticeable about the sixth week; reached its maximum during the eighth to tenth week; and remained unaltered from then on. These eye changes were limited to animals on diets containing little iodine, in whom hyperplasia of the thyroid glands, followed later on in some cases by atrophy, was found. Apart from changes in the parathyroid glands the state of other endocrine organs is not mentioned.

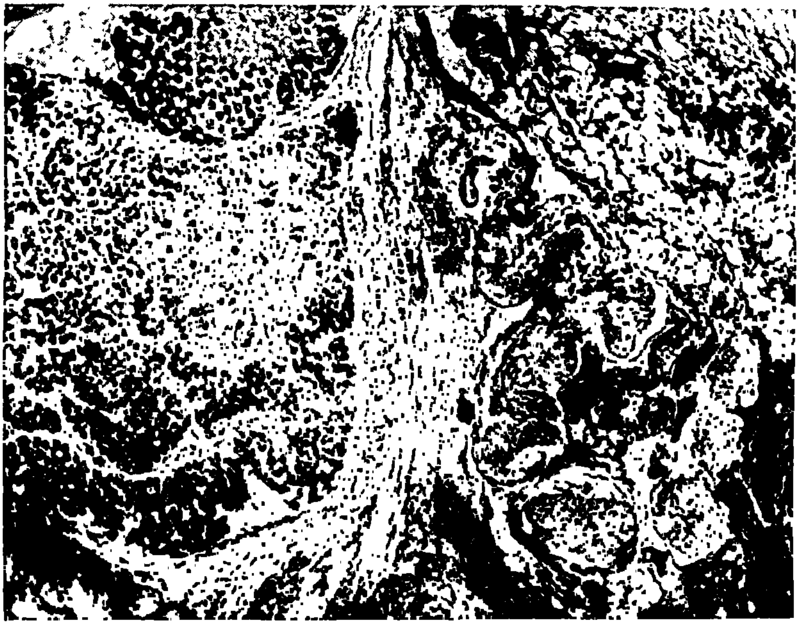

FIG. 3. Section of the left orbital tissues of the rat with asymmetrical proptosis. There is no evidence of inflammation or tumour present. (Formalin-Zenker, Hematoxylin-Eosin, $7\mu \times 75$).

Heinemann (1937) produced exophthalmos in guinea pigs and rats by injecting thyrotropic hormone (Schering-Kahlbaum). At autopsy the thyroids were shown to be enlarged. Exophthalmos occurred in animals treated for more than 2 weeks, and was most marked in those animals which had been treated longest. There was no loss of weight, but loss of fur was noted. The adrenal glands in the exophthalmic guinea pigs were definitely enlarged.

Definite exophthalmos persisting after death has been reported by Rawson, quoted by Means (1944), in rats in which a hyperplastic goitre was produced by treatment with sulfathiazole. Sellers and Ferguson (1949) have also observed proptosis in rats fed with propylthiouracil for prolonged periods. It could be seen in some animals after only 3 months, but it took 7 months before it was present in almost every rat. It was best observed if the animal was active and alert. All rats were under weight and had scanty, coarse hair. The weight of the eyeballs was not larger than in the control group, and no lymphocytic infiltration was present in the retrobulbar tissues.

It is generally stated that exophthalmos is due to excessive production of the thyrotropic hormone. The occurrence of thyroid changes in the experiments just quoted could be interpreted as supporting this thesis. Since the prolonged administration of cortisone or ACTH is said to induce thyroid depression—the "corticogenic hypothyroidism" of Wolfson *et al.* (1950, 1951)—the occurrence of proptosis in cortisone-treated animals could be explained on the basis of a subsequent increase in the secretion of the thyrotropic hormone. Some clinical (Richardson *et al.* 1950; Reiss *et al.* 1950; Werner, 1950); and experimental (Winter *et al.*, 1950) findings do not, however, support this view. Since it has furthermore been shown that cortisone significantly increases the thyrotropin-induced exophthalmos in normal as well as in thyroidectomised guinea pigs (Aterman, 1952b, c), it is difficult to maintain the thesis that this cortisone effect is mediated through the thyroid. Even if cortisone can affect the thyroid as an indirect consequence of some change in the pituitary, it is also probable that the hormone acts directly on the connective tissues of the body. The explanation of the ocular protrusion observed in the present experiments would then be the changed physical state of these tissues within the orbit (Aterman 1952b, c).

Reference has already been made to the unusual course of exophthalmos in rat No. 30 (Fig. 2). No explanation can be advanced for the exceptional features present in this animal. Since an increase, no change, or a decrease in the intra-ocular tension under the influence of ACTH or cortisone have been reported under various clinical and experimental conditions (Bardram, 1950; Fitzgerald *et al.* 1951; Olson *et al.* 1951; Sallman *et al.* 1952; Stewart Duke-Elder, 1951), it is questionable whether the increased tension present in the proptosed left eye of this rat can be attributed to a direct effect of cortisone or whether it was the result of the pronounced exophthalmos. The marked asymmetry of the latter also remains unaccounted for. Although some of the rats in the present series developed quite a marked degree of exophthalmos, we have not been able to reproduce a similar picture. Unilateral exophthalmos in experimental animals has, however, been reported by other workers (Friedgood, 1934; Smelser, 1937; Thompson, 1933). The absence of any macroscopic or microscopic evidence of a retroorbital tumor or inflammation (Fig. 3) in our experimental animal suggests a similarity with the rare but well known cases of "unilateral" endocrine exophthalmos in man occasionally mistakenly diagnosed as retroorbital tumors and even operated on (Anderson, 1947; Brain, 1936, Bedrossian, 1951; Burch, 1929, 1942; Denis and Mahaux, 1951; Drescher and Benedict, 1950; Jensen, 1940; Kisner and Mahorner, 1947; Klotz, 1948; Regnier, 1942; Rundle and Wilson, 1944, 1945; Saltzmann and Mellicker, 1951). That endocrine factors should lead to asymmetrical changes in symmetrical organs is a puzzling, but not unknown phenomenon of which several instances can be found in the literature (Bond, 1914; Greene, 1948; Peyton, 1942; Wilkins, 1948).

SUMMARY

The occurrence of varying degrees of bilateral proptosis has been observed in young male Wistar rats treated for 4 weeks with cortisone acetate. In one animal marked asymmetry of the proptosis occurred. These findings are discussed in the light of our present knowledge of the etiology of exophthalmos.

Acknowledgment

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NITROGEN BALANCE, LIVER PROTEIN REPLETION AND BODY COMPOSITION OF CORTISONE TREATED RATS

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IN 1940, Long, Katzin and Fry demonstrated that the administration of adrenal cortical extract to fasting rats caused a loss of nitrogen in the urine and an increase in liver glycogen. They suggested a relationship between protein breakdown and glycogen synthesis in such animals. For reviews of subsequent investigations, the reader is referred to those of Ingle (1950), Sayers (1950) and Engel (1951).

When cortisone acetate[1] became available in 1949, it seemed desirable to investigate further the effects of cortisone on nitrogen balance, on liver protein regeneration, and on the distribution of protein and fat in the body.

EXPERIMENTS AND RESULTS

Male albino rats of the Carworth strain were selected from single shipments and grouped to obtain equivalent average body weights. Adrenalectomies were performed by the lumbar approach under nembutal anesthesia and operated rats were given 1% saline to drink. All animals were housed in individual mesh-bottom cages in air conditioned quarters during the experiments. Nitrogen was determined by the micro-Kjeldahl method. References to other analytical techniques accompany the data.

The probability (p) of erroneously concluding that there are significant differences between means was calculated by Student's t test or from the variance ratio (F). See Snedecor (1946).

a) *Nitrogen balance.* Rats weighing 250 gm., on the average, were fed protein-free diet (Silber and Porter, 1950), *ad libitum* for 7 days. On the 8th, 9th, and 10th days, consumption of the diet was limited to 8 gm./rat /day and each of thirty-two rats was given daily subcutaneous injections of 3 mg. cortisone acetate in aqueous suspension. A second group of thirty-two rats did not receive cortisone. On the 9th day, all rats were placed in metabolism cages in groups of two. On this and the subsequent day, half of the cortisone-treated rats and half of the untreated rats each received, in addition to the daily allotment of protein-free diet, an oral dose of a solu-

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[1] Cortisone Acetate (Merck).

tion of amino acids or egg albumin (300 mg. N/kg. rat). Twenty-four hours after the second dose, the rats were given 10 ml. of water orally, and urines were collected with cage washings two hours later. The urinary nitrogen data are summarized in Table 1.

TABLE 1. THE EFFECT OF CORTISONE ACETATE UPON NITROGEN BALANCE. NITROGEN GIVEN, 300 MG./KG./DAY

Group	Treatment		Av. rat wts. before test, gm.	3-day wt. loss, gm.	Liver wt., % body wt.	Av. urinary N, mg./kg./day ± s.e.
	Cortisone	Amino acids				
			Amino acid experiment			
1–4	+	+	226	11.5	4.1	474 ± 7.2
5–8	+	0	225.5	15.5	3.7	365 ± 24.1
9–12	0	+	227	2.0	3.2	372 ± 5.4
13–16	0	0	226	8.0	3.0	267 ± 9.7
			Egg albumin experiment			
	Cortisone	Egg albumin				
1–4	+	+	243	—	—	367 ± 7.2
5–8	+	0	239	—	—	338 ± 25.0
9–12	0	+	239	—	—	272 ± 17.3
13–16	0	0	238	—	—	231 ± 6.4

Sixty-four per cent of the orally administered amino acid nitrogen was retained by the cortisone-treated rats and 65% by the controls. The retention of egg albumin nitrogen also was comparable in the two groups, 90% and 86%, respectively. Expressed as nitrogen balance indices (Allison, Seeley, and Ferguson, 1947) these values would be 0.64, 0.65, 0.90, and 0.86. Thus, cortisone had no effect on the retention of ingested nitrogen. The rats lost 13 gm. body weight/kg. and 100 mg. urinary nitrogen/kg./day more than their controls. Nevertheless, the livers of the rats given cortisone contained 200 mg. more nitrogen/kg. body weight than those of the controls.

b) *Liver protein repletion.* Rats which were adrenalectomized on the fourth day of protein depletion were supplied protein-free diet for six additional days. On the 11th, 12th, and 13th days some were allowed to consume 4.5 gm. protein-free diet, others 4.5 gm. 20% casein diet per 100 gm. body weight each day. Cortisone injections (1.5 mg./100 gm. body wt.) were made daily from the fourth to the thirteenth day, inclusive. Intact rats were treated similarly.

No significant difference was observed between the intact and the operated rats so the data were pooled, as shown in Table 2. The livers of the repleted, cortisone-treated rats were larger and contained more protein nitrogen, more NPN and more glycogen. Serum protein concentrations

TABLE 2. THE EFFECTS OF CORTISONE ON LIVERS OF RATS DURING PROTEIN-REPLETION

	Cortisone-treated (13 rats)	Controls (9 rats)
Liver wt., gm./100 gm. rat	5.28 ±0.16	3.18 ±0.061
Protein N. mg./100 gm. rat	127.8 ±1.7	94.6 ±2.0
N. P. N., mg./100 gm. rat	17.9 ±0.35	13.0 ±0.27
Glycogen, mg./100 gm. rat	429.8 ±36	48.8 ±5.9

were also increased (6.6 versus 5.7 gm.%). Similar but less striking differences were seen in the rats which remained on protein-free diet throughout the experiment.

The subsequent experiment was designed to study the quantitative relationship between the effects of cortisone and protein feeding upon liver protein regeneration. Adrenalectomized rats were fed protein-free diet *ad libitum* from the fourth through the seventh day after operation. They were then allowed to consume 5 gm. per 100 gm. body weight per day of protein-free or 15% casein diet, and some were given daily subcutaneous injections of cortisone acetate (2 mg./100 gm. rat). Rats were sacrificed two or four days after the first injection. Analysis of variance of the data (Table 3) shows that the very significant effects of cortisone and of dietary protein upon liver nitrogen had no influence upon one another.

c) *Effects of cortisone on carcass and viscera of rats.* From the above results, it was clear that cortisone could cause an increase in liver and serum protein while nitrogen was being lost in the urine, whether or not the animals were supplied dietary protein. The following experiment was designed to locate the source of the endogenous nitrogen available for liver regeneration and for excretion.

Five days after adrenalectomy, groups of five rats were injected daily with 0.1 or 1.0 mg. cortisone acetate per 100 gm. rat, and allowed to consume 3.5 gm. of protein-free diet per 100 gm. rat/day. Control animals were not included because adrenalectomized rats do not survive on the protein-free diet in spite of saline therapy.

The rats were sacrificed after 14 days. They were exsanguinated, and the carcasses were divided as follows: (1) liver, (2) spleen, (3) gastro-intestinal organs (washed stomach and intestines, and adhering structures), (4) urogenital organs (testes, prostate, kidneys, bladder), (5) pleural organs (heart and lungs), (6) carcass (muscle and bone, including the head and brain), and (7) skin and hair.

Livers and spleens were homogenized with water and aliquots taken for the various analyses. The other tissues were macerated in a Waring Blender with 70% alcohol, then extracted in Soxhlet extractors with 95% ethanol, then ether. The extracts were evaporated to dryness and the residues

TABLE 3. EFFECT OF PROTEIN AND CORTISONE UPON LIVER N REGENERATION IN ADRENALECTOMIZED RATS

5 rats/group; saline *ad libitum*

Protein	Cortisone	Days	Body wt. at start, gm.	Body wt. at sac., gm.	Liver N, mg./100 gm. rat ±s.e.
0	0	2*	190	178	85.9±2.76
0	0	4*	190	168	84.3±1.40
0	+	2	190	181	101.8±1.18
0	+	4	190	172	110.1±3.39
+	0	2	190	189	99.0±2.84
+	0	4	190	193	102.4±2.06
+	+	2	190	183	126.4±1.68
+	+	4	190	186	125.3±3.50
		0	190	190	92.2±2.40

* These group consumed 3.5 and 2.5 gm. diet/100 gm. rat/day. The remaining groups consumed all 5 gm. of their diets/100 gm. rat/day.

ANALYSIS OF VARIANTS

Source of variation	Degrees of freedom	Mean squares	F*	p
Protein	1	3766.57	100.79	<0.005
Cortisone	1	6352.60	169.99	<0.005
Days	1	59.86	1.60	
Protein × Cortisone	1	56.77	1.52	
Protein × Days	1	14.95	0.40	
Cortisone × Days	1	21.60	0.58	
Protein × Cortisone × Days	1	156.24	4.18	0.05
Error	40	37.37		

* F for 1 and 40 d.f., $(p=0.005)=8.83$; $(p=0.05)=4.08$.

partitioned between water and petroleum ether. The defatted tissues were dried and pulverized, and aliquots were taken for analysis, except in the case of skin, where the whole sample was used. For protein analyses samples of dried defatted carcasses and the skins were extracted with N/10 NaOH. Three 24-hour extractions at room temperature were sufficient to remove essentially all of the soluble proteins from the carcass samples. The skins were extracted four times, with freezing and thawing, to remove the soluble proteins. Collagen in carcasses and skins was determined essentially according to Lowry, Gilligan and Katersky (1941). Total nitrogen on the residues from the collagen extractions of the carcasses is reported here as "elastin" N; the figure also includes bone nitrogen. Since the hair was not separated from the skins, total nitrogen on skin residues was not determined.

The rats which received the higher dose of cortisone lost significantly more weight than those receiving the lower dose (Table 4). The carcasses and skins lost weight roughly in proportion to body weight. The internal organs maintained or increased in weight, so the organ weight to body weight ratios showed significant increases. The analytical data on blood

TABLE 4. ORGAN WEIGHTS, SERUM PROTEIN AND LIVER ANALYSES. ADRENALECTOMIZED PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Initial body wt., gm.	229	229	
Body wt. loss, %	27.3 ± 0.96	30.8 ± 0.33	<0.025
Carcass wet wt., gm./100 gm. rat	60.2 ± 0.83	58.0 ± 0.59	<0.1
Dry, defatted, carc. wt., gm./100 gm. rat	16.8 ± 0.20	16.4 ± 0.38	>0.2
Skin and hair wet wt., gm./100 gm. rat	18.1 ± 0.38	17.3 ± 0.21	<0.2
Dry, defatted skin wt., gm./100 gm. rat	6.08 ± 0.18	6.06 ± 0.08	>0.2
Liver wt., gm./100 gm. rat	2.93 ± 0.07	4.18 ± 0.12	<0.005
Liver dry wt., gm./100 gm. rat	0.75 ± 0.022	1.14 ± 0.042	<0.005
Viscera wt., gm./100 gm. rat	11.3 ± 0.29	12.7 ± 0.51	<0.05
Dry, defatted viscera wt., gm./100 gm. rat	1.31 ± 0.024	1.46 ± 0.055	<0.05
Total serum protein, gm. %*	5.27 ± 0.16	6.00 ± 0.071	<0.005
Albumin (pooled sample), gm. %	1.45	2.12	
Globulin (pooled sample), gm. %	3.72	3.93	
A/G ratio	0.39	0.54	
Liver protein N, mg./100 gm. rat	76.5 ± 1.31	105.5 ± 1.86	<0.005
Liver NPN, mg./100 gm. rat	9.47 ± 0.30	13.51 ± 0.34	<0.005
Liver carbohydrate, mg./100 gm. rat	137 ±23.4	320 ±20.5	<0.005
Liver lipid (alc.-ether sol.) mg./100 gm. rat	261 ±22.3	452 ±24.6	<0.005
Liver desoxyribonucleic acid, mg./100 gm. rat†	15.2 ± 0.39	17.1 ± 1.26	<0.2
Liver ribonucleic acid, mg./100 gm. rat‡	43.8 ± 3.56	68.4 ± 4.10	<0.005

* Total protein and protein fractions by the method of Kibrick and Blonstein (1948).
† Dische (1930).
‡ Davidson and Waymouth (1944).

serum showed that cortisone prevented a decrease or caused an increase in albumin, even though these animals were consuming protein-free diet, but there was little change in globulin.

As the livers increased in size under the influence of the higher dose of cortisone, protein and fat (alcohol-ether soluble) increased in about equal proportions while carbohydrate increased to a greater extent. Liver ribonucleic acid increased in proportion to liver weight, but desoxyribonucleic acid remained essentially constant in quantity, indicating an increase in cytoplasm without an increase in the number of cells. (The relative increase in desoxyribonucleic acid indicated in the table was of doubtful significance, $p<0.2$, and was at least partially a result of the decrease in body weight.)

The distribution of nitrogen in the animals (Table 5) closely paralleled the organ and body weight distribution discussed above (Table 4). Total nitrogen of the rats decreased proportionately with body weight loss. However, there was an excessive loss of nitrogen from the carcass whereas the liver, gastro-intestinal organs and urogenital organs were spared nitrogen loss. The collagen and elastin nitrogen of the carcass showed a relative and absolute increase, while the more labile alkali-soluble nitrogen fraction decreased significantly. The fraction of nitrogen contributed by the skin remained the same in the two groups.

The ratio of total fat (Table 6) to body weight increased by 50%

TABLE 5. NITROGEN DISTRIBUTION IN ADRENALECTOMIZED, PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Nitrogen, % of rat total N (skin elastin and hair omitted)			
Carcass	68.0 ±0.49	65.0 ±0.43	<0.01
Skin	22.1 ±0.45	22.6 ±0.32	>0.2
Liver	3.03 ±0.085	4.49 ±0.114	<0.005
Spleen	0.30 ±0.055	0.25 ±0.019	>0.2
Pleura	0.25 ±0.004	0.25 ±0.007	>0.2
Gastro-intestinal	3.66 ±0.070	4.27 ±0.130	<0.01
Urogenital	2.55 ±0.073	2.96 ±0.120	<0.05
Total N gm./100 gm. rat	2.79 ±0.061	2.67 ±0.037	>0.2
N of carcass fractions, % of carcass total N			
Water solubles from Soxhlet extract	5.84 ±0.28	6.77 ±0.27	<0.1
Alkali soluble	82.30 ±0.57	76.87 ±0.48	<0.01
Collagen	10.16 ±0.54	14.24 ±1.00	<0.025
Elastin & bone	1.67 ±0.088	2.11 ±0.12	<0.05
Carcass total N, gm./100 gm. rat	1.90 ±0.053	1.74 ±0.031	<0.1
N of skin fractions, % of skin total N (hair & elastin omitted)			
Water soluble from Soxhlet extract	6.84 ±0.34	5.84 ±0.33	<0.2
Alkali soluble	41.56 ±0.53	41.16 ±1.12	>0.2
Collagen	51.48 ±0.88	52.90 ±1.17	>0.2
Soluble N + Collagen N, gm./100 gm. rat	0.62 ±0.017	0.61 ±0.001	>0.2

($p < 0.1$) under the influence of cortisone. Particularly striking increases in fat were observed in the carcass and organs of the pleural cavity and less striking increases in the urogenital organs and livers. Fat in the skin and gastro-intestinal organs remained essentially constant.

DISCUSSION

Since the report of Long, Katzin and Fry (1940) cortisone has frequently been termed a "catabolic" hormone. From isotope experiments, Hoberman (1950) and Clark (1950) have considered cortisone to exert catabolic or

TABLE 6. FAT* IN ADRENALECTOMIZED PROTEIN-DEPLETED RATS. TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	*p*
Carcass, gm. fat/100 gm. rat	1.46 ±0.14	2.62 ±0.35	<0.025
Skin, gm. fat/100 gm. rat	0.91 ±0.22	1.03 ±0.09	>0.2
Liver, mg. fat/100 gm. rat	115 ±11.4	158 ±20.8	<0.2
Pleura, gm. fat/100 gm. rat	41.5 ±5.9	95.3 ±11.8	<0.005
Gastro-intestinal, mg. fat/100 gm. rat	317 ±159	588 ±137	>0.2
Urogenital, mg. fat/100 g. rat	451 ±101	731 ±81	<0.1
Spleen, mg. fat/100 gm. rat	14.2 ±2.5	16.3 ±1.3	>0.2
Total fat, gm./100 gm. rat	3.30 ±0.62	5.24 ±0.63	<0.1

* Benzin soluble.

anti-anabolic effects on protein metabolism. Ingle (1950) and Engel (1951) have suggested that the primary effect of cortisone may be a decrease in the utilization of carbohydrate. The fact that cortisone in milligram quantities can depress the body weight of rats without a proportionate decrease in food intake is well substantiated. From the observations of Kochakian and Robertson (1951) and from the present work, it is clear that the loss of body weight is due to loss of substance, including nitrogen, from the carcass rather than from the internal organs.

Loss of nitrogen from the carcass was not accompanied by deficient utilization of ingested protein as evidenced by nitrogen balance and liver protein repletion. This is in agreement with the report of Wells and Kendall (1940) that adrenalectomy did not influence the utilization of exogenous protein.

Engel, Schiller, and Pentz (1949) found that in nephrectomized rats, exogenous amino acids or glucose spared the breakdown of endogenous protein caused by injection of adrenal cortical extract. In the present experiments, however, the effect of cortisone upon the incorporation of nitrogen in the liver or its excretion in the urine was not influenced by the ingestion of minimal amounts of protein by intact rats. In rats fed normal amounts of dietary protein Clark (in press) found that cortisone treatment caused an increase in the excretion of isotopic nitrogen fed as glycine. The apparent contradiction between this and the present work may be related to the level of nitrogen intake.

In the nitrogen balance experiment it was noted that the body weights of the rats given cortisone decreased 13 gm./kg./day more than their controls in spite of equivalent caloric intakes. If the tissue protein is assumed to contain 16% nitrogen and the wet tissue to contain 20% protein, only one-fifth of the weight loss can be related to the loss of nitrogen in the urine. There was no evidence in these experiments that dehydration occurred in the carcasses of the cortisone-treated rats although they did excrete larger volumes of urine. They also consumed more water, and dry weight data showed that the water concentrations in the carcasses were comparable in the treated and untreated groups.

The liver protein of cortisone-treated rats decreased in concentration, but increased in total, This may be related to the deposition of glycogen and the increase in cell size. However, the elevated serum albumin concentrations found in the cortisone-treated rats also indicates an increase in protein synthesis by the liver. Mushett, Porter and Silber (unpublished) have found that cortisone-treated dogs on stock diet *ad libitum* showed changes similar to those observed in rats, i.e., in liver protein, fat, and nucleic acid. Friedgood, Vars and Zerbe (1950) have reported that cortisone facilitated liver regeneration after partial hepatectomy. Kochakian and